Helsinki University Biomedical Dissertations No. 35

REGULATION OF STAT5 ACTIVATION

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ACADEMIC DISSERTATION

To be publicly discussed, with the permission of the Faculty of Science of the University of Helsinki, in the Large Lecture Hall of the Haartman Institute, Haartmaninkatu 3, Helsinki, on September 26th, 2003, 12 o'clock.

HELSINKI 2003

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ISSN 1457-8433
ISBN 952-10-1316-8 (Printed version)
ISBN 952-10-1317-6 (PDF version)
http://ethesis.helsinki.fi
Yliopistopaino
Helsinki 2003

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ORIGINAL PUBLICATIONS

The thesis is based on the following original articles, which are referred to by their Roman numerals in the text.

I. Valgeirsdóttir S, Paukku K, Silvennoinen O, Heldin C-H, and Claesson-Welsh L. Activation of Stat5 by platelet-derived growth factor (PDGF) is dependent on phosphorylation sites in PDGF β-receptor juxtamembrane and kinase insert domains.
Oncogene, **16**, 505-515, 1998

II. Paukku K, Valgeirsdóttir S, Saharinen P, Bergman M, Heldin C-H, and Silvennoinen O. Platelet-derived growth factor (PDGF)-induced activation of signal transducer and activator of transcription (Stat) 5 is mediated by PDGF β-receptor and is not dependent on c-Src, Fyn, Jak1 or Jak2 kinases.
Biochemical Journal, **345**, 759-766, 2000

III. Paukku K, Yang J, and Silvennoinen O. Tudor and nuclease-like domains containing protein p100 functions as a coactivator for Stat5.
Molecular Endocrinology, **17**, 1805-1814, 2003

IV. Peltola K, Paukku K, Silvennoinen O, and Koskinen PJ. Pim-1 kinase inhibits Stat5-dependent transcription via its interactions with SOCS1 and SOCS3.
Submitted

ABBREVIATIONS

CBP	CREB-binding protein
C/EBPβ	CCAATT/enhancer binding protein-β
CIS	cytokine-inducible SH2-containing protein
Dex	dexamethasone
EGF	epidermal growth factor
EMSA	electrophoretic gel-mobility shift assay
EPO	erythropoietin
GAS	γ-activated site
GH	growth hormone
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GST	glutathione S-transferase
IFN	interferon
IL	interleukin
IGF-1	insulin-like growth factor-1
ISGF-3	interferon-stimulated gene factor-3
Jak	Janus kinase
kDa	kilodalton
MAPK	mitogen-activated protein kinase
NF-κB	nuclear factor κB
Nmi	N-myc interactor
PDGF	platelet-derived growth factor
PIAS	protein inhibitor of activated Stats
PRL	prolactin
RTK	receptor tyrosine kinase
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SH	Src homology
SIE	*sis*-inducible element
SN	staphylococcal nuclease
SOCS	suppressor of cytokine signalling
Spi	serine protease inhibitor
Stat	signal transducer and activator of transcription
TAD	transactivation domain
TD	tudor domain

SUMMARY

Signal transducers and activators of transcription (Stats) comprise a family of transcription factors that are activated by various cytokines and growth factors. Seven mammalian Stat proteins exist and they all share a similar overall structure. Stat5 was originally identified as a transcription factor that regulates the β-casein gene in response to prolactin (PRL), but is activated also by several other ligands, including growth hormone, erythropoietin, interleukin-2 (IL-2), IL-3, and platelet-derived growth factor (PDGF). Stat proteins are latent in the cytoplasm until activated through receptor-mediated tyrosine phosphorylation leading to dimerization and nuclear translocation. In the nucleus, Stat dimers bind to DNA and initiate transcription of the target genes. In the case of cytokine receptors, Stat activation most often utilizes kinases of the Jak family. However, the mechanism of Stat activation in growth factor-stimulated signaling has remained elusive. In addition to the PDGF receptor (PDGFR), which is a tyrosine kinase itself, also cytoplasmic tyrosine kinases of Jak and Src families are activated upon PDGF stimulation. In our studies it was observed that the kinase responsible for Stat5 activation is the PDGFR kinase itself.

The molecular mechanisms that underlie Stat5-mediated transcription are not fully understood, but these mechanisms involve interactions and cooperation with sequence specific transcription factors as well as with transcriptional coregulators. In mammary cells, the synergistic activation of transcription of the β-casein gene has been shown to be dependent on the interaction between Stat5, glucocorticoid receptor, and CCAATT/enhancer binding protein-β. Recently, the expression of a tudor and nuclease-like domains containing coactivator protein p100 was found to be abundant in the nuclei of mammary epithelial cells. The protein levels of p100 increased in response to lactogenic hormones during lactation and correlated with the induction of β-casein gene expression. These findings suggested a role for p100 in lactation. Our results showed that p100 interacts with Stat5 and enhances the Stat5-mediated transcriptional activation, thus indicating that p100 functions as a coactivator for Stat5. In addition, PRL up-regulates p100 protein levels in mouse mammary epithelial cells. These results suggest the existence of a positive regulatory loop in PRL-induced transcription, where PRL stabilizes p100 protein, which in turn can cooperate with Stat5 in transcriptional activation.

Stat activation is inhibited by dephosphorylation, but also by induced suppressors of cytokine signalling (SOCS) proteins, which can block Stat activation by inactivation of Jak, by blocking access of Stat to the receptor sites, or by targeting bound proteins to proteasomal degradation. Stat5 induces the expression of numerous proteins including β-casein, Cyclin D, Bcl-xL, Pim-1, c-Fos and c-Jun. Pim-1 is a serine/threonine kinase, which has been shown to be a survival factor for IL-3-dependent cells. In our studies IL-3-dependent FDCP myeloid cells that have enforced expression of Pim-1 exhibited inhibited Stat5 DNA-binding and tyrosine phosphorylation. Pim-1 was not able to phosphorylate Stat5 or directly bind to Stat5. Instead, Pim-1 cooperated with SOCS-1 and SOCS-3 to inhibit Stat5 activity. SOCS-3 and Pim-1 associated *in vivo* and Pim-1 phosphorylated SOCS-3. Our data suggest that Pim-1 together with SOCS-1 and SOCS-3 are components of a negative feedback loop that allows Stat5 to inhibit its own activation.

INTRODUCTION

1. Jak/Stat pathway

Majority of the mammalian signal transduction processes are initiated as a result of interactions between ligand and receptor (reviewed in Kishimoto, 1994). These interactions result in biochemical changes, which are processed and delivered to the nucleus to cause changes in gene expression. Investigations of transcriptional responses to interferons (IFNs) led to the identification of Janus kinase-Signal transducer and activator of transcription (Jak/Stat) pathway. During the past decade, a large amount of evidence has accumulated to indicate that cytokines transmit their signals via a family of Jak tyrosine kinases and their downstream targets, Stats (reviewed in Darnell *et al.*, 1994; Ihle, 1995; Schindler and Darnell, 1995; Ihle *et al.*, 1997). Stats were originally described as ligand-induced transcription factors in IFN-treated cells (reviewed in Darnell *et al.*, 1994; Darnell, 1997). Subsequent studies showed that Stats play a critical role in signal transduction pathways associated with the large hematopoietic family of cytokines. Stats are also activated after activation of receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (Leaman *et al.*, 1996; Vignais *et al.*, 1996; Valgeirsdottir *et al.*, 1998). In addition, Stats are activated by hormones (like growth hormone; GH) (Silva *et al.*, 1994; Bergad *et al.*, 1995), and also the stimulation of several members of G-protein-coupled receptors have been shown to lead to Stat activation (Marrero *et al.*, 1995; Wong and Fish, 1998; Vila-Coro *et al.*, 1999). Stat proteins are initially present in inactive forms in the cytoplasm. In the general model for the activation of Jak/Stat pathway, the activation cascade starts with the activation of receptor-bound Jak kinases following ligand stimulation and receptor dimerization (Figure 1). Jak kinases subsequently phosphorylate receptor at tyrosine residues. This phosphorylation recruits Stats to the receptor, after which Stats are phosphorylated, dimerized, and quickly translocated to the nucleus. In the nucleus, the dimers bind to specific sequences called γ-activated sequences (GAS) in the promoter regions of their target genes, and stimulate the transcription of these genes (reviewed in Schindler and Darnell, 1995; Darnell, 1997).

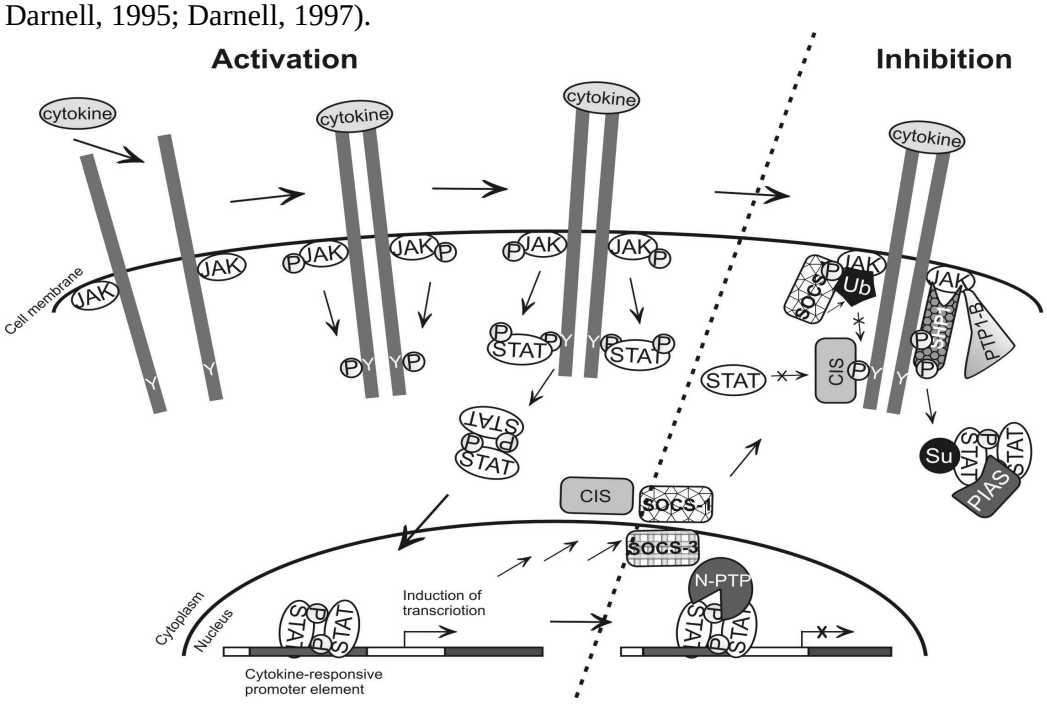

Figure 1. Activation and inhibition of the Jak/Stat pathway. Cytokine binds to its receptor resulting in receptor dimerization, transphosphorylation of Jaks, and phosphorylation of the receptor. Stats bind to the phosphorylated receptor, are in turn phosphorylated, dimerize, and enter the nucleus where they initiate transcription. Some of the genes activated are SOCS family members (CIS, SOCS-1 and SOCS-3 in the figure), which in turn bind and deactivate Jaks and occupy receptor Stat-binding sites. SOCS proteins can also target proteins to degradation by ubiquitin-proteasome system (Ub). Tyrosine phophatase SHP-1 binds to the activated receptor before dephosphorylating the Jaks. A cytosolic protein-tyrosine phosphatase PTP1B also binds and deactivates Jaks. A nuclear phosphatase for Stats is indicated as N-PTP in the figure. PIAS molecules modulate the activity of Stat dimers by preventing them from binding to DNA or inducing sumoylation (Su).

1.1. Members of Stat family

Seven mammalian Stats exist (Stat1, 2, 3, 4, 5A, 5B and 6). Stat homologues have been identified also in a number of more primitive organisms, where they appear to transduce developmentally important signals. To date, homologs to mammalian Stats have been identified in amoebae (*Dictyostelium discoideum*), nematode (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), malaria mosquito (*Anopheles gambiae*), frog (*Xenopus laevis*) and zebrafish (*Danio rerio*) (Aubry and Firtel, 1999; Barillas-Mury *et al.*, 1999; Oates *et al.*, 1999; Zeidler *et al.*, 2000; Pascal *et al.*, 2001). Recently, a Stat homologous to Stat5 was identified in pufferfish (*Tetraodon fluviatilis*) (Sung *et al.*, 2003). In *Drosophila*, the Jak/Stat pathway is genetically well characterized and has been shown to be important for growth, development, and differentiation of cells and tissues (Hou *et al.*, 1996; 2002; Yan *et al.*, 1996a; 1996b). In mammals, the seven Stats are segregated to three clusters. Each cluster represents a tandem duplication. In mouse, Stat1 and Stat4 map to chromosome 1, while Stat2 and Stat6 are located in chromosome 10. Stat3 and Stat5 are located in chromosome 11, but the Stat5 gene has undergone an additional duplication (i.e. Stat5A and Stat5B) more recently during evolution (Copeland *et al.*, 1995).

Stat1 and Stat2

Research on IFN signaling pathways led to the discovery of the first members of Stat family, namely Stat1 and Stat2 (Fu, 1992; Fu *et al.*, 1992; Schindler *et al.*, 1992; Shuai *et al.*, 1992; Pearse *et al.*, 1993; Veals *et al.*, 1993; Shuai, 1994). IFNγ induces formation of Stat1 homodimers, which initiate transcription of GAS-driven genes. IFNα and IFNβ lead to formation of Stat1 homodimers and also of Stat1/Stat2 heterodimers, which associate with the IRF-9 (p48) protein to form a complex called IFN-stimulated gene factor-3 (ISGF-3), and induce transcription of IFN-stimulated response element (ISRE)-driven genes. IFNγ activates Stat1 almost exclusively, and mice that lack Stat1 have no innate response to either viral or bacterial pathogens, because defence against these pathogens usually requires response to IFN (Durbin *et al.*, 1996; Meraz *et al.*, 1996). Although a number of cytokines and growth factors can activate Stat1, currently there are no major developmental deficits identified in these mice attributable to non-IFN signalling (Table 1). Stat1-deficient mice develop spontaneous and chemically-induced tumors more readily than wild-type animals, defining Stat1 as a tumor suppressor (Kaplan *et al.*, 1998a). There is evidence that Stat1 activation commonly leads to antiproliferative and proapoptotic events, and this may partly explain why the lack of this protein *in vivo* leads to increased tumor formation (reviewed in Bromberg, 2000). Additionally, Stat1 controls the expression of proteins involved in antigen presentation, thereby affecting the immunogenicity of the tumor. The low levels of Stat1 in the nucleus of unstimulated cells suggest that Stat1 might cooperate with other transcriptional factors to regulate the basal expression of certain genes (reviewed in Schindler and Strehlow, 2000).

Despite being the second Stat cloned, Stat2 remains least well understood. Stat2 represents a unique member of the Stat family, being the only Stat that does not bind to GAS elements following activation. Like Stat1-deficient mice, also mice lacking Stat2 are fertile, viable and develop normally (Park *et al.*, 2000). However, similar to Stat1-deficient mice, Stat2-null mice are susceptible to viral infections. This is due to impaired IFNα/β responsiveness, supporting the idea that Stat2 plays an essential role in the ISGF-3 complex induced by IFNα (Table 1).

Stat3

Stat3 is ubiquitously expressed in most tissues and early during post-implantation (Duncan *et al.*, 1997). Stat3 was originally identified as an acute-phase response factor, activated by IL-6, but Stat3 is also activated by many other cytokines (Akira *et al.*, 1994). Stat3 is also involved in transformation. Stat3 protein is found activated in many cancerous cell lines and tumors (Bowman *et al.*, 2000). Disruption of the *stat3* gene leads to an early embryonic lethal phenotype (Takeda *et al.*, 1997). Tissue-specific targeting of Stat3 has not been found to have major developmental consequences; however, other abnormalities are apparent (Table 1). Stat3-deficient T-cells and hepatocytes exhibit a poor response to IL-6 (Takeda *et al.*, 1998; Alonzi *et al.*, 2001), whereas Stat3 deficiency in macrophages and neutrophils is associated with exaggerated production of cytokines presumably due to impaired IL-10 responsiveness (Takeda *et al.*, 1999). Stat3-null mammary glands exhibit a significant delay in programmed cell death that occurs during cyclical mammary gland involution (Chapman *et al.*, 1999). Finally, tissue-specific deletion of Stat3 in keratinocytes leads to defects in both skin and hair (Sano *et al.*, 1999).

Stat4

Stat4 was identified by screening of cDNA libraries and sequence databases for homologues of Stat1 and Stat2 (Yamamoto *et al.*, 1994). In contrast to other Stats, which are ubiquitously expressed, the expression of Stat4 was found to be limited to natural killer (NK) -cells, dendritic cells and T-lymphocytes. Stat4 is activated by IL-12, which plays a critical role in the development of the Th1 subset of T helper cells (versus Th2 cells). Stat4 knockout mice confirmed the important role of Stat4 in IL-12 signaling (Thierfelder *et al.*, 1996; Kaplan *et al.*, 1998b). As might be predicted, Stat4-deficient mice are resistant to autoimmune diseases characterized by a Th1 response (Chitnis *et al.*, 2001) (Table 1). On the other hand, Stat4-deficient mice have increased susceptibility to infection with intracellular organisms (Tarleton *et al.*, 2000). Stat4 was recently shown to be activated also by IL-23 (Parham *et al.*, 2002).

Stat5

Stat5 (Stat5A) was first identified as a PRL-induced mammary gland factor (MGF) (Gouilleux *et al.*, 1994). Subsequent studies led to a discovery of two closely related sequences, Stat5A and Stat5B (Azam *et al.*, 1995; Mui *et al.*, 1995). These proteins share over 90% identity and diverge only at their carboxy (C)-terminus. In addition to PRL, Stat5 proteins are activated by many other cytokines and growth factors (Ihle, 1996; Darnell, 1997; Leonard and O'Shea, 1998). Both Stat5 proteins are ubiquitously expressed, but the expression profiles are different. Stat5A is the predominant form in mammary gland, whereas Stat5B is more prominently expressed in liver. *In vitro* studies with dominant negative Stat5 mutants demonstrated that Stat5A and Stat5B display functional redundancy (Moriggl *et al.*, 1996). Despite these structural and functional similarities, Stat5A and Stat5B single knockout mice had remarkably distinct phenotypes (Table 1). Stat5A knockout mice have impaired mammary gland development due to loss of PRL responsiveness (Liu *et al.*, 1997; Teglund *et*

al., 1998), whereas Stat5B-deficient mice have sexually dimorphic growth retardation due to defects in GH signaling (Udy *et al.*, 1997; Teglund *et al.*, 1998). The Stat5A/B double knockout mice exhibited a more severe phenotype. Many of the double knockout mice die within a few weeks of birth, are infertile, and have defective mammary gland development (Miyoshi *et al.*, 2001) (Table 1). In addition, these mice are small and have reduced size of fat pads and reduced levels of insulin-like growth factor-1 (IGF-1). Stat5A/B double knockout mice have also hypocellular bone marrow, lymphopenia, neutrophilia, modest anemia, thrombocytopenia, reduced numbers of B-cell precursors and mature B-cells, and reduced responsiveness to IL-7 (Sexl *et al.*, 2000; Bunting *et al.*, 2002; Snow *et al.*, 2002). In addition, NK-cells are absent, but thymic development is intact; however, peripheral T-cells from these mice express activation markers constitutively and have impaired *in vitro* proliferation (Matsumoto *et al.*, 1999). Activated Stat5 has been found in several malignancies (Weber-Nordt *et al.*, 1996; Gouilleux-Gruart *et al.*, 1997; Yu *et al.*, 1997; Bovolenta *et al.*, 2002). In hematopoietic cells, Stat5 has been demonstrated to play a critical role in regulating apoptosis (Onishi *et al.*, 1998; Teglund *et al.*, 1998; Nosaka *et al.*, 1999; Socolovsky *et al.*, 1999), and in promoting proliferation and cell cycle progression (Moriggl *et al.*, 1999; Nieborowska-Skorska *et al.*, 1999). More information about Stat5 is presented in chapter 2.

Stat6

Stat6 is expressed in all tissues and was originally purified from the cell extracts as the IL-4-stimulated Stat (Hou *et al.*, 1994). Stat6 functions in the adaptive immune system and is critical for Th2 differentiation (Hou *et al.*, 1994). Stat6 is also activated by IL-13, which shares a receptor chain with IL-4 (Lin *et al.*, 1995). IL-4 and IL-13 play an important role in regulating acquired immunity. In line with this, Stat6 knockout mice exhibit profound defects in their ability to develop Th2-cells (Kaplan *et al.*, 1996; Shimoda *et al.*, 1996; Takeda *et al.*, 1996) (Table 1). Stat6 is required for induction of IL-4-dependent gene expression leading to Th2-cell differentiation and IL-4-dependent B-cell proliferation (Shimoda *et al.*, 1996; Takeda *et al.*, 1996). Additionally, Stat6-deficient B-cells are unable to undergo class switching and produce IgE (Shimoda *et al.*, 1996). Stat6 may be necessary for the down-regulation of tumor immunosurveillance, since Stat6 deficiency is associated with resisted tumor reappearance (Terabe *et al.*, 2000).

ROLE OF STAT PROTEINS AS REVEALED BY GENE-TARGETING STUDIES IN MICE	
Stat1	Viable and fertile, impaired responses to interferons (IFNα/β and IFNγ); increased susceptibility to tumors
Stat2	Viable and fertile; impaired responses to interferons (IFNα/β), reduced Stat1 expression in some tissues
Stat3	Embryonic lethal. Conditional knockouts have multiple defects in adult tissues including impaired cell survival and impaired response to pathogens
Stat4	Viable and fertile, impaired Th1 differentiation due to loss of IL-12 responsiveness
Stat5A	Viable and fertile, impaired mammary gland development due to loss of PRL responsiveness
Stat5B	Viable and fertile, impaired growth due to loss of GH responsiveness
Stat5A/B	Viable, female infertility, defective mammary gland development, reduced body mass in males and females, defective T-cell proliferation
Stat6	Viable and fertile, impaired Th1 differentiation due to loss of IL-4 responsiveness

Table 1. Phenotypes of Stat-deficient mice, adapted from Levy and Darnell, 2002; O'Shea *et al.*, 2002.

1.2. Stat structure

Stats are proteins of 750 to 850 amino acids that contain six structurally and functionally conserved domains (Figure 2). The amino (N)-terminal domain is conserved among the Stats and represents an independently folded and stable moiety (Vinkemeier *et al.*, 1996). The crystal structure of the N-terminus of Stat4 reveals a dimer (Vinkemeier *et al.*, 1998). Several studies suggest that this N-terminal dimerization promotes cooperativity of binding to tandem GAS elements (Vinkemeier *et al.*, 1996; Xu *et al.*, 1996b; Vinkemeier *et al.*, 1998). Studies have also suggested that the N-terminal domain regulates nuclear translocation of Stats (Strehlow and Schindler, 1998) and can promote interaction with the transcriptional coactivator CBP(CREB-binding protein)/p300 (Zhang *et al.*, 1996), the PIAS family proteins (Shuai, 2000), and receptor domains (Leung *et al.*, 1995; Li *et al.*, 1997; Murphy *et al.*, 2000).

The coiled-coil domain consists of four α-helices. The crystal structures of Stat1 and Stat3 reveal that this domain forms a large predominantly hydrophilic surface that is available for specific interactions with other helical proteins (Becker *et al.*, 1998; Chen *et al.*, 1998). Proteins interacting with the coiled-coil domain are many and include IRF-9, the transcription factor c-Jun, N-myc interactor (Nmi), and Stat3-interacting protein (StIP1) (Zhang *et al.*, 1999b; Zhu *et al.*, 1999; Collum *et al.*, 2000; Horvath, 2000). In addition, the coiled-coil domain is also implicated in receptor binding, tyrosine phosphorylation, and nuclear export (Begitt *et al.*, 2000; Zhang *et al.*, 2000).

Figure 2. The domain structure of Stat contains six structurally and functionally conserved domains: N-terminal domain, coiled-coil domain, DNA-binding domain, linker domain, SH2 domain, and transactivation domain.

The DNA-binding domain is a region of β-sheet structures connected by unstructured loops (Chen *et al.*, 1998). Each Stat component of the dimer recognizes bases in the most proximal half GAS element. The cooperativity in DNA-binding is likely to be important in effective transcriptional activity, because the number of direct contact sites between amino acid residues and DNA are modest, accounting for dissociation constant in the nanomolar range. Interestingly, the conformation of DNA-binding domain is different before and after ligand stimulation and may therefore have still unknown functions (McBride *et al.*, 2000). The linker domain connects the DNA-binding domain with the SH2 domain. The observations done with Stat1 crystal structure suggest that DNA-binding capacity can be regulated by structural changes in the SH2 domain as it binds to phosphotyrosine at the C-terminus of another Stat protein (Chen *et al.*, 1998) (Figure 3). The linker domain of Stat1 is also implicated in transcriptional regulation (Yang *et al.*, 1999). The SH2 domain is the most highly conserved Stat domain. Stat dimerization depends on the interaction between the SH2 domain of one Stat monomer and the tyrosine phosphorylated tail segment of the other monomer. The ability of SH2 domain to recognize specific phosphotyrosine motifs plays an essential role in several Stat signaling events; Stat homo- or heterodimerization, recruitment to the cytokine receptor through recognition of specific receptor phosphotyrosine motifs, and association with the activating Jak (reviewed in Shuai *et al.*, 1994). All Stats except Stat2

have been shown to form stable homodimers. Additionally, many Stats can heterodimerize with other Stats through SH2-phosphotyrosine interaction (reviewed in Schindler and Darnell, 1995). Although the Stat5 proteins do not heterodimerize with less related Stats, consistent with their SH2 domains more homologous overall than other Stats, it is logical that Stat5A and Stat5B form both homo- and heterodimers (Liu *et al.*, 1996).

The C-terminal transactivation domain (TAD) is poorly conserved among the Stats. This is in line with the ability of Stat-TAD to regulate unique transcriptional responses. The evidence that the C-terminus encodes TAD came from a comparative analysis of the full-length Stat1 and an alternatively spliced isoform Stat1, which lacks the last 38 C-terminal amino acids (Schindler *et al.*, 1992). The C-terminally truncated Stat1 was not able to drive transcription. C-terminally truncated isoforms have also been identified for Stat3, Stat4, Stat5, and Stat6 (Schindler and Strehlow, 2000; Sherman *et al.*, 2002; Hoey *et al.*, 2003), and they appear to function as dominant-negative regulators. The TAD region contains serine residue(s) that can modulate the transcriptional activity of Stats (reviewed in Decker and Kovarik, 2000). The TAD region has been characterized to be important in coregulator recruitment (Bhattacharya *et al.*, 1996; Zhang *et al.*, 1996; Pfitzner *et al.*, 1998; Litterst and Pfitzner, 2002).

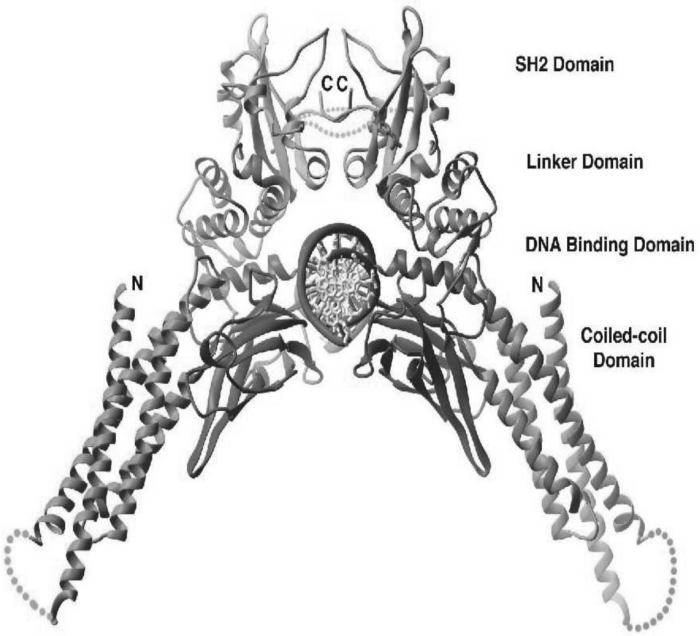

Figure 3. The crystal structure of Stat1 homodimer in a complex with the DNA. Stat1 utilizes a DNA-binding domain with an immunoglobulin fold. The Stat1 dimer forms a C-shaped clamp around DNA that is stabilized by interactions between the SH2 domain of one monomer and the tyrosine-phosphorylated C-terminal segment of the other monomer. The phosphotyrosine-binding site of the SH2 domain in each monomer is coupled structurally to the DNA-binding domain, suggesting a potential role for the SH2-phosphotyrosine interaction in the stabilization of DNA-interacting elements. Reprinted from Chen *et al.*, 1998, with permission from Elsevier, Copyright (1998).

1.3. Receptors and tyrosine kinases involved in Stat activation

1.3.1. Cytokine receptors

A family of soluble polypeptides, referred to as cytokines, controls hematopoietic cell proliferation and differentiation. Cytokines include interleukins (ILs), IFNs, colony-stimulating factors, and erythropoietin (EPO) (reviewed in Metcalf, 1989). Hematopoietic cytokine receptors are transmembrane glycoproteins, composed of an extracellular ligand-binding domain, a hydrophobic transmembrane part, and an intracellular domain (reviewed in Ihle *et al.*, 1994a; Silvennoinen *et al.*, 1997) (Figure 4). Cytokine receptors can be organized into subgroups by the number of receptor subunits and the use of common signaling chains. Type I cytokine receptors include the gp130 family, the common γ chain (γc) family, the common β chain (βc) family, and the single chain family of the receptors (reviewed in Schindler and Strehlow, 2000). Type II cytokine receptors include receptors for IFNs and also receptors for IL-10 and IL-10-related cytokines (reviewed in Kotenko and Pestka, 2000). Type I receptors share similar basic structural features with each other (reviewed in Davies and Wlodawer, 1995; Leonard and Lin, 2000), and are characterized by the presence of four conserved cysteine residues, a WSXWS motif, and fibronectin type III modules in the extracellular domain. In addition, a Box1/Box2 region exists in the cytoplasmic domain.

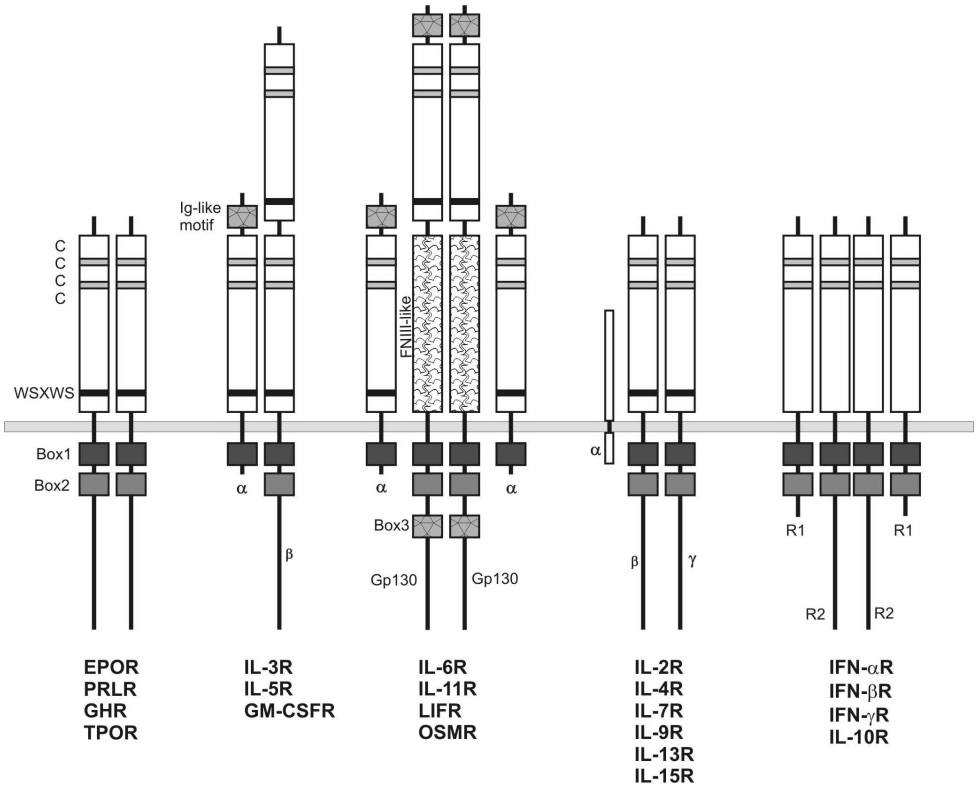

Figure 4. The general structure of receptors of the hematopoietic cytokine receptor family. The extracellular domains of type I cytokine receptors are characterized by a homologous region called the cytokine or hematopoietic receptor homology (CHR) domain. The N-terminus of the CHR usually contains two pairs of conserved cysteine residues, and the C-terminus contains a conserved WSXWS motif. The type II cytokine receptors have the similar extracellular domain structure as type I receptors, but they differ by a unique cysteine distribution and lack of the WSXWS motif. The cytoplasmic regions of the cytokine receptors are less conserved than the extracellular regions. The membrane-proximal parts show limited sequence similarity. The two conserved regions are referred as Box1 and Box2 motifs.

Members of the gp130 receptor family can be divided into two subclasses. One includes receptors for IL-6, IL-11, leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and neurotrophin-1/B-cell-stimulating factor-3 (NNT-1/BSF-3), which share a common receptor glycoprotein subunit called gp130 (reviewed in Hirano *et al.*, 1994; Kishimoto, 1994; Taga, 1996). Receptors for G-CSF, leptin and IL-12 belong to the second group of the gp130 receptors, which are characterized by the employment of at least one gp130-like receptor chain. The shared gp130 receptor chain associates with ligand-specific subunits (i.e. α-chains) to form functionally specific receptors. A receptor for the recently characterized heterodimeric cytokine IL-23 is composed of IL-12R β-chain and a novel cytokine receptor subunit, IL-23R (Frucht DM, 2002). Other gp130 family members signal predominantly through Stat3, while IL-12- and IL-23-induced gene expressions are mediated mainly through Stat4 activation (reviewed in Akira, 1997; Ihle *et al.*, 1997; Lankford and Frucht, 2003; Schindler and Strehlow, 2000) (Table 2).

The γc family consists of receptors for IL-2, IL-4, IL-7, thymic stromal lymphopoietin (TSLP), IL-9, IL-13, IL-15, and IL-21 (reviewed in Demoulin and Renauld, 1998; Murata *et al.*, 1998). Each of these receptors is composed of at least one specific ligand-binding chain (usually referred to as the α-chain) and a shared receptor component, the γc. Some receptors (e.g. for IL-2 and IL-15) include a third chain. While the receptors for IL-2, IL-7, TSLP, IL-9, IL-15 and IL-21 transmit signals mainly through activation of Stat5, the receptors for IL-4 and IL-13 activate Stat6 more predominantly (reviewed in Demoulin and Renauld, 1998) (Table 2).

The βc family of cytokine receptors include receptors for granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-3, and IL-5 (reviewed in Miyajima *et al.*, 1993). These receptors are a family of heterodimeric transmembrane proteins expressed by myeloid lineage cells. Each of these receptors has a unique ligand-binding α-chain and a shared βc. Signal transduction by these receptors is mediated primarily through activation of Jak2 and Stat5 (reviewed in Schindler and Strehlow, 2000) (Table 2).

The family of single chain receptors consists of receptors for GH, EPO, PRL and thrombopoietin (TPO) (reviewed in Goffin and Kelly, 1997; Yoshimura and Misawa, 1998). The single chain receptors consist of one chain that homodimerizes upon ligand binding. They signal mainly through sequential activation of Jak2 and Stat5 (Gouilleux *et al.*, 1995; Nagata and Todokoro, 1995) (Table 2).

Type II cytokine receptors include: IFNγ, IFNαs/β/ω/limitin, IL-10, IL-19, IL-20, IL-22, and IL-24. Type II cytokine receptors consist of at least two different subunits, which are both used in signaling, but only one binds the cytokine (reviewed in Kotenko and Pestka, 2000). The IFNα receptor associates with Jak1 and Tyk2 to mediate IFN-dependent gene transcription. This entails the activation of two distinct Stat-dependent signaling pathways, ISGF-3 and Stat1 homodimers. Stat3 homodimers and Stat1/Stat3 heterodimers are also activated, but their role in signaling remains to be elucidated. Cellular responses following IFNγ stimulation are mediated through the recruitment of Stat1 to the IFN receptor 1 (IFNR1) chain and its subsequent activation by Jak1/Jak2 (Greenlund *et al.*, 1994; 1995). The IL-10 receptor signals through sequential activation of Tyk2/Jak1 and Stat1/Stat3 (Finbloom and Winestock, 1995). IL-22 is an IL-10-related cytokine, which induces activation of Stat1, Stat3, and Stat5 (Lejeune *et al.*, 2002). IL-20 appears to signal through Stat3 (Blumberg *et al.*, 2001) (Table 2).

LIGAND	JAKs ACTIVATED	STATs ACTIVATED
IFN family		
IFNα/β/ω/Limitin	Jak1, Tyk2	Stat1, Stat2, Stat3-Stat6
IFN-γ	Jak1, Jak2	Stat1, Stat2, Stat5
IL-10	Jak1, Tyk2	Stat1, Stat3
IL-19, IL-20, IL-24	?	Stat1, Stat3
IL-22	Jak1, Tyk2	Stat1, Stat3, Stat5
Gp130 family		
IL-6, OSM, LIF,	Jak1, Jak2	Stat1, Stat3, Stat5
IL-11, CNTF, CT-1, NNT-1/BSF-3	Jak1, Jak2	Stat1, Stat3
G-CSF	Jak1, Jak2	Stat1, Stat3, Stat5
Leptin	Jak2	Stat1, Stat3, Stat4, Stat5
IL-12	Jak2, Tyk2	Stat1, Stat3, Stat4, Stat5
IL-23	Jak2, Tyk2	Stat1, Stat3, Stat4, Stat5
γc family		
IL-2	Jak1, Jak3	Stat1, Stat3, Stat5
IL-7	Jak1, Jak3	Stat1, Stat5
IL-9	Jak1, Jak3	Stat3, Stat5
IL-15	Jak1, Jak3	Stat5, Stat6
TSLP	none	Stat5
IL-21	(Jak1), Jak3	Stat3, Stat5 (Stat1)
IL-4	Jak1, Jak3	Stat3, Stat5, Stat6
IL-13	Jak1, Jak2, Tyk2	Stat3, Stat5, Stat6
βc family		
IL-3, IL-5, GM-CSF	Jak2	Stat5 (+Stat1, Stat3, Stat6 for IL-3)
Single chain family		
EPO, PRL	Jak2	Stat1, Stat3, Stat5
GH	Jak1, Jak2	Stat1, Stat3, Stat5
TPO	Jak2, Tyk2	Stat1, Stat3, Stat5
Growth factors		
EGF	Jak1, Jak2	Stat1, Stat3, Stat5
PDGF	Jak1, Jak2, Tyk2	Stat1, Stat3, Stat5, Stat6
CSF-1	Tyk2, Jak1	Stat1, Stat3, Stat5
HGF	Jak2	Stat1, Stat3, Stat5
VEGF	?	Stat1, Stat3, Stat5, Stat6
Insulin	none	Stat1, Stat3, Stat5
IGF-1	Jak1, Jak2, Tyk2	Stat3
SCF	Jak2	Stat1, Stat5
FGF	Jak2	Stat1, Stat3
G-proteins		
Angiotensin II AT1	Jak2, Tyk2	Stat1, Stat2, Stat5
LH	Jak2	Stat1, Stat5
Serotonin	Jak2	Stat3

Table 2. Cytokines, growth factors and G-proteins and their substrate Jaks and Stats. Abbreviations: OSM, oncostatin M; LIF, leukemia inhibitory factor; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; NNT-1/BSF-3, neurotrophin-1/B-cell-stimulating factor-3; TPO, thrombopoietin; and TSLP, thymic stromal lymphopoietin. Adapted from Grimley *et al.*, 1999; Schindler and Strehlow, 2000.

1.3.1.1. Stat activation by cellular tyrosine kinases

Molecular cloning of cytokine receptors and subsequent structure-function studies have revealed that unlike growth-factor receptors, cytokine receptors lack a cytoplasmic kinase domain. However, the interaction of a cytokine with its receptor induces rapid tyrosine phosphorylation of the the receptor and a variety of cellular proteins, suggesting that these receptors transmit their signals through cellular tyrosine kinases (reviewed in Ihle, 1994). Cytokine receptors have been shown to rely on members of the Jak family of tyrosine kinases to provide this activity (reviewed in Ihle *et al.*, 1994b). Jaks associate also with the receptor tyrosine kinases and with G-protein-coupled receptors (Marrero *et al.*, 1995; Sasaguri *et al.*, 2000). There are four members of the Jak family in mammals, Jak1, Jak2, Jak3, and Tyk2 ranging in molecular weight from 110 to 140 kDa. Jak1, Jak2 and Tyk2 are ubiquitously expressed, whereas the expression of Jak3 is more restricted. Jak3 is expressed in the cells of hematopoietic and lymphoid lineages, in vascular smooth muscle cells, and in endothelium (Kawamura *et al.*, 1994; Rane and Reddy, 1994; Musso *et al.*, 1995; Tortolani *et al.*, 1995; Gurniak and Berg, 1996; Verbsky *et al.*, 1996). Jaks are usually constitutively associated with the membrane proximal domain of the cytokine receptors (reviewed in Ihle, 2001). Ligand binding promotes the dimerization of receptors into an active conformation (reviewed in Wells and de Vos, 1996). The activation is believed to lead to close approximation of cytoplasmic receptor tails, enabling the transphosphorylation (i.e. activation) of the receptor-associated Jaks. Activated Jaks then phosphorylate specific tyrosine motifs present in the receptor intracellular domains, which in turn recruit Stats and other SH2 domain-containing signaling molecules to the receptor.

Figure 5. The domain structure of Jak kinases. Jak kinases possess a kinase domain (JH1) at the C-terminus, a pseudokinase domain (JH2), an SH2-like domain (JH3-JH4), and a FERM domain (JH4-JH7).

Jak sequence consists of seven regions of high homology, JH1-JH7, where JH1 has been shown to encode a kinase domain and JH2 a pseudokinase domain (Figure 5). The conserved kinase domain exhibits classical features of a tyrosine kinase (reviewed in Hubbard and Till, 2000), while the pseudokinase domain has all the structural features of a *bona fide* tyrosine kinase, but it lacks catalytic activity. There is growing evidence that the pseudokinase domain regulates the activity of the kinase (Luo *et al.*, 1997; Barahmand-Pour *et al.*, 1998; Saharinen and Silvennoinen, 2002; Saharinen *et al.*, 2003). The N-terminal Jak region is somewhat divergent between family members. The N-terminus has been implicated in receptor association and appears to play an important role in determining the specificity of this binding (Leonard and O'Shea, 1998). The JH3-JH4 region shares some similarity with SH2 domains, and the JH4-JH7 region constitutes a FERM domain (Four-point-one, Ezrin, Radixin, Moesin) (Girault *et al.*, 1998; Kampa and Burnside, 2000). Notably, the FERM domain was identified in the interactions between Jaks and other kinases (Miyazaki *et al.*, 1998; Zhu *et al.*, 1998). In addition, the FERM domain of Jak1 may be critical for interaction with the gp130 receptor chain (Hilkens *et al.*, 2001). Recent studies suggested a novel role for the Jak3 FERM domain in maintaining a functional kinase domain (Zhou *et al.*, 2001).

In addition to Jak kinases, also several other tyrosine kinases are activated by cytokine and growth factor receptors. Src-family members such as Src, Lck, Hck, Lyn, Fyn, and Fgr have been linked to Stat activation (Lund *et al.*, 1997; Yu *et al.*, 1997; Schreiner *et al.*, 2002). However, it has remained somewhat unclear whether these kinases are able to activate Stats directly or whether they function e.g. through Jak kinases (Campbell *et al.*, 1997). Other kinases implicated in Stat activation include: Etk/Bmx, a tyrosine kinase of Tec family

kinases, which is able to activate Stat1, Stat3 and Stat5 (Saharinen *et al.*, 1997; Wen *et al.*, 1999), the focal adhesion kinase (FAK), which can activate Stat1 (Xie *et al.*, 2001), and the nonreceptor tyrosine kinase Fes, which has the ability to bind and activate Stat3 in response to GM-CSF (Park *et al.*, 1998).

1.3.2. Oncogenic tyrosine kinases in Stat activation

In contrast to normal signaling, in which Stat activation is rapid and transient and depends on ligand stimulation, constitutive signaling by Stats has been associated with malignant progression. In tumor cells, constitutive activation of Stats is linked to persistent activity of tyrosine kinases. Such oncogenic tyrosine kinases are often activated as a consequence of permanent ligand/receptor engagement in autocrine or paracrine cytokine and growth factor signaling or represent autonomous constitutively active enzymes as a result of genetic alterations found in tumor but not in normal cells. In mammalian cells, the original report demonstrating that Stats are constitutively activated in cells stably transformed by a specific oncoprotein, linked activation of the oncogenic Src tyrosine kinase to the activation of Stat3 (Yu *et al.*, 1995). A TEL/Jak2 fusion (fusion of the Jak2 protein to the Ets family transcription factor TEL) has been shown to result in Stat activation and myeloproliferative disease (Ho *et al.*, 1999; Lacronique *et al.*, 2000), and both TEL/Jak2 and TEL/Abl induce the activation of Stat1 and Stat5 in human leukemia (Wilbanks *et al.*, 2000). In addition, both the cell transformation by oncogenic tyrosine kinase v-Abl and the expression of Bcr-Abl oncogenic fusion protein, lead to the activation of Stat1 and Stat5 in chronic myelogenous leukemia (Danial *et al.*, 1995; Carlesso *et al.*, 1996). Fibroblast transformation by v-Fps correlates with potent activation of endogenous Stat3 (Garcia *et al.*, 1997). Also the expression of the anaplastic lymphoma kinase (ALK) induces constitutive phosphorylation of Stat3 (Zamo *et al.*, 2002). In addition to cellular oncogenes, Stat5 is activated by oncogenic receptor tyrosine kinases or oncogenic fusions of the receptors like the TEL/PDGFβR and Huntingtin interacting protein 1 (HIP1)/PDGFβR (Okuda *et al.*, 1996; Ross *et al.*, 1998; Ross and Gilliland, 1999; Wilbanks *et al.*, 2000; Sternberg *et al.*, 2001). Stat5 is also activated in fish melanoma cells by a melanoma-inducing EGFR-related receptor Xmrk (Morcinek *et al.*, 2002).

CELL TYPE	ONCOGENIC PROTEINS	ACTIVATED STATs
Fibroblast cells	v-Src, c-Src	Stat3
	v-Fps (Fes)	Stat3
	v-Sis (PDGF)	Stat3
	Polyoma virus middle T antigen	Stat3
	v-Ros	Stat3
	IGF-1 receptor	Stat3
	c-Eyk, v-Eyk	Stat1, Stat3
Myeloid cells	v-Src	Stat1, Stat3, Stat5
T-cells	Lck	Stat3, Stat5
Mammary/lung epithelial cells	v-Src	Stat3
	Etk/Bmx	Stat1, Stat3, Stat5
Gallbladder adenocarcinoma cells	v-Src	Stat3
Pre-B lymphocytes	v-Abl	Stat1, Stat5
Erythroleukemia/blast cells/Basofils/mast cells	Bcr-Abl	Stat1, Stat5
Primary bone marrow cells	Bcr-Abl	Stat5

Table 3. Stat activation by oncogenic kinases, modified from Bowman *et al.*, 2000.

1.3.3. Receptor tyrosine kinases

RTKs are composed of an extracellular ligand binding domain, followed by a single transmembrane domain and a cytoplasmic domain containing a conserved protein tyrosine kinase core, flanked by regulatory sequences (reviewed in Hubbard and Till, 2000) (Figure 6). Ligand binding to RTKs promotes receptor dimerization and subsequent activation of intrinsic tyrosine kinase activity that results in transphosphorylation of specific tyrosine residues (reviewed in Weiss and Schlessinger, 1998). Phosphorylation of a conserved tyrosine residue inside the kinase domains leads to an increase in the catalytic efficiency of the RTK. The phosphorylated tyrosine residues located outside the kinase domain serve as docking sites for an array of intracellular signaling molecules containing SH2 domains (reviewed in Pawson, 2002). These activated proteins then initiate signaling cascades resulting in activation of transcription factors that determine a variety of cell responses, including mitogenesis, migration, and differentiation.

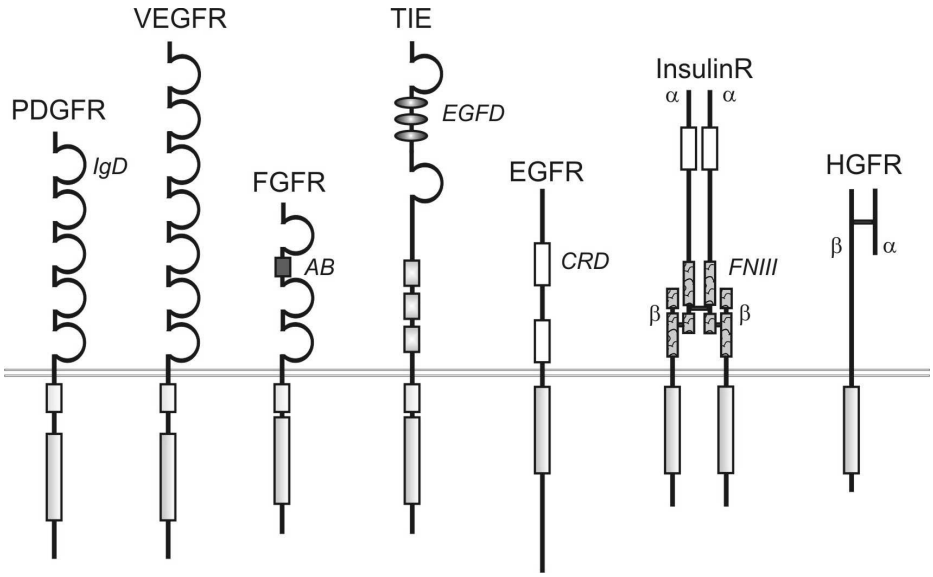

Figure 6. The human RTK families involved in the activation of Stats. RTKs are composed of an extracellular ligand-binding domain, followed by a single transmembrane domain and a cytoplasmic domain containing a conserved protein tyrosine kinase. The prototypic receptor for each family is indicated above the receptor, and the known members are listed below. Abbreviations of the prototypic receptors: PDGFR, platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor; TIE, tyrosine kinase receptor; EGFR, epidermal growth factor receptor; InsulinR, insulin receptor; HGFR, hepatocyte growth factor receptor. Other abbreviations: IgD, immunoglobulin-like domain; AB, acidic box; EGFD, epidermal growth factor-like domain; CRD, cystein-rich domain; FNIII, fibronectin type III-like domain. Adapted from Blume-Jensen and Hunter, 2001.

RTKs for growth factors, such as receptors for PDGF, EGF, stem cell factor (SCF), hepatocyte growth factor (HGF), insulin, vascular endothelial growth factor (VEGF), and IGF-1, have been implicated in Stat activation. PDGFs exert their effects on target cells by

activating two structurally related tyrosine kinase receptors. Each α- and β-receptor (PDGF α-R and PDGF β-R) contains five immunoglobulin-like (Ig-like) domains in their extracellular part, and an intracellular tyrosine kinase domain that contains a characteristic inserted sequence without homology to kinases (Escobedo and Williams, 1988) (Figure 6). PDGFR activates Stat1, Stat3, Stat5, and Stat6 (Choudhury *et al.*, 1996; Patel *et al.*, 1996; Vignais *et al.*, 1996; Valgeirsdottir *et al.*, 1998) (Table 2). Same set of Stat proteins is activated by endothelial receptor tyrosine kinases (VEGFRs) 1-3, which resemble PDGFRs in structure (Pajusola *et al.*, 1992; Terman *et al.*, 1992; Enaida *et al.*, 1999; Korpelainen *et al.*, 1999; Bartoli *et al.*, 2000) (Figure 6). The second family of endothelial RTKs consists of Tie-1 and Tie-2 (Tek) (Partanen *et al.*, 1992; Dumont *et al.*, 1993; Ziegler *et al.*, 1993). The FGF family of receptors comprises of four structurally related members, FGFR1, FGFR2, FGFR3, and FGFR4. These receptors also exhibit Ig-like domains in their structure (Johnson and Williams, 1993; Partanen *et al.*, 1993), and activate Stat1 and Stat3 (Hart *et al.*, 2000; Sahni *et al.*, 2001). The EGF (ErbB) RTK family consists of four homologous members; EGFR (ErbB1), ErbB2 (Neu), ErbB3, and ErbB4 (reviewed in Boulougouris and Elder, 2001) (Figure 6), which activate Stat1, Stat3, and Stat5 (Ruff-Jamison *et al.*, 1993; 1994; Leaman *et al.*, 1996; Gallego *et al.*, 2001). The insulin receptor (InsR) is a heterotetrameric membrane protein that consists of two identical α- and β- subunits (reviewed in Czech, 1985; Canivet *et al.*, 1992) (Figure 6). The activation of InsR results in activation of Stat1, Stat3, and Stat5 (Ceresa and Pessin, 1996; Chen *et al.*, 1997; Velloso *et al.*, 1998). Stat3 is activated by HGFR (MET) (Schaper *et al.*, 1997; Boccaccio *et al.*, 1998), which is the prototypic member of a subfamily of heterodimeric receptor tyrosine kinases including also receptors called Ron and Sea (reviewed in Comoglio and Boccaccio, 1996). The structure of these receptors is characterized with a highly glycosylated and entirely extracellular α-subunit, as well as a β-subunit with a large extracellular region, a membrane-spanning segment, and an intracellular tyrosine kinase domain (Weidner *et al.*, 1993; Zhen *et al.*, 1994) (Figure 6).

1.3.3.1. PDGF and PDGFR

PDGF is made up as a homo- or heterodimer of disulfide-bonded polypeptide chains. The chains are synthesized as precursor molecules that undergo proteolytic processing (Ostman *et al.*, 1991). PDGF is a major mitogen for fibroblasts, smooth muscle cells, and many other cells, and PDGF has been shown to regulate cell proliferation, survival and chemotaxis *in vitro* (reviewed in Heldin and Westermark, 1999). PDGF- and PDGFR-targeted mice have shown that PDGF has important functions as a paracrine growth factor in development (Leveen *et al.*, 1994; Bostrom *et al.*, 1996; Betsholtz and Raines, 1997). Overactivity of PDGF has been linked to different types of disorders, including atherosclerosis, fibrotic diseases, and malignancies (reviewed in Heldin and Westermark, 1999). The transforming oncogene *sis* encodes a PDGF-like growth factor, which transforms cells by an autocrine mechanism. For almost two decades, PDGF-AA, -BB and -AB were thought to be the only members of the PDGF family. Recently, two additional PDGF polypeptide chains were discovered, namely PDGF-C and PDGF-D (Ding *et al.*, 2000; Li *et al.*, 2000; Bergsten *et al.*, 2001; LaRochelle *et al.*, 2001). Dimeric PDGF isoforms dimerize receptors by binding two receptors simultaneously (Bishayee *et al.*, 1989; Heldin *et al.*, 1989), resulting in αα-receptor and ββ-receptor homodimers, and αβ-receptor heterodimers. These three receptor combinations transduce overlapping but not identical cellular signals. The phosphorylation of a conserved tyrosine residue inside the kinase domains (Tyr849 in α-R and Tyr857 in β-R) leads to an increase in the catalytic activity of the kinases (Kazlauskas and Cooper, 1989). A large number of SH2 domain proteins have been shown to bind to docking sites in α- and β-receptors (reviewed in Williams *et al.*, 1991; Heldin *et al.*, 1998).

Some of these proteins are enzymes, like the members of tyrosine kinase families of Src and Jak, tyrosine phosphatase SHP-2, and a GTPase-activating protein (GAP) for Ras, whereas other molecules like Grb2, Grb7, Nck, Shc, and Crk have adapter functions (reviewed in Heldin, 1997).

2. Stat5

2.1. Regulation of Stat5 activity

The Jak/Stat pathway is important for many responses including differentiation, proliferation, and oncogenesis. It is, therefore, not surprising that numerous regulatory layers exist to modulate this signaling pathway. The effect of the negative and positive regulatory processes determines the rate at which Stat signals are transduced. Stat signals are down-regulated at several points in the signaling cascade including the receptors, Jaks, and the Stat molecules themselves.

2.1.1. Mutations and deletions

A constitutively active Stat5A mutant (Stat5A1*6), which renders IL-3-dependent cell lines IL-3-independent, was found by screening randomly mutated Stat5As (Onishi *et al.*, 1998). In addition to inducing IL-3-independent proliferation, this constitutively active Stat5 induced apoptosis and differentiation in the same cell line after IL-3 stimulation (Nosaka *et al.*, 1999). Stat5A1*6 showed markedly elevated DNA-binding and transcriptional activities with stable tyrosine phosphorylation and nuclear accumulation. Stat5A1*6 harbors two point mutations; one in the TAD and other in the DNA-binding domain. The same results as with the 1*6 mutant Stat5A were obtained with the 1*6 mutant Stat5B (Onishi *et al.*, 1998), indicating that the biological activities of the mutant Stat5A are not specific to the 1*6 mutant of Stat5A and will reflect physiological functions of Stat5. Another constitutively active Stat5 mutant (Stat5A-Asn642His) has an asparagine-to-histidine mutation very close to the phosphotyrosine-binding site in the SH2 domain and has identical phenotype as the mutant Stat5A1*6 (Ariyoshi *et al.*, 2000). The mutation in Stat5A-Asn642His resulted in restoration of the conserved critical histidine, which is involved in the binding of phosphotyrosine in the majority of SH2-containing proteins. *Pim-1* and *bcl-xL* are genes induced by Stat5 (Lilly and Kraft, 1997; Packham *et al.*, 1998; Thomas *et al.*, 1998) and were expressed even in the absence of IL-3 in Ba/F3 cells expressing Stat5A-Asn642His. It is likely that this constitutive induction of genes important for proliferation and differentiation induces IL-3-independent cell growth of the Ba/F3 cells as well as in Ba/F3 cells expressing Stat5A1*6 (Nosaka *et al.*, 1999). Altogether, it seems that the activation of Stat5A1*6 and Stat5A-Asn642His mutants mimics the physiological activation of Stat5.

Functionally distinct C-terminally truncated Stat isoforms have been reported. Naturally occurring truncated Stat1 and Stat3 bind DNA but fail to activate transcription as independent factors (Muller *et al.*, 1993; Caldenhoven *et al.*, 1996). Also naturally occurring C-terminally truncated Stat5 isoforms have been identified in several different cell types (Azam *et al.*, 1995; Rosen *et al.*, 1996; Kirken *et al.*, 1997). A number of studies have clearly shown that the truncated Stat5 is the predominant phosphorylated Stat5 form observed after IL-3, GM-CSF, or EPO stimulation in early hematopoietic cells (Azam *et al.*, 1995; Mui *et al.*, 1995; Rosen *et al.*, 1996). Significantly, these results were observed in both mice and humans, and with both primary cells and established cell lines. As compared to the primary forms of Stat5A and Stat5B, with molecular weights of 94 kDa (Stat5Aα) or 92 kDa (Stat5Bα), shortened Stat5 variants (Stat5Aβ and Stat5Bβ) exhibit molecular weight of

approximately 80kDa. As a general feature, truncated Stat5 isoforms lack a functional TAD and act as dominant negative inhibitors of Stat5-dependent transcription. They remain tyrosine phosphorylated and bound to GAS sites for longer periods of time than the full-length Stat5 isoforms, suggesting that the C-terminal sequences may affect the interaction with a tyrosine phosphatase (Moriggl *et al.*, 1996; 1997). In addition, it has been speculated that these variants are able to inhibit transcription by recruiting nuclear corepressors to gene promoters (Nakajima *et al.*, 2001). Previous studies have shown that the truncated Stat5 proteins are unable to transactivate *cis* and o*sm* (Mui *et al.*, 1996; Wang *et al.*, 1996); two target genes shown to be up-regulated by Stat5 in early hematopoiesis. Azam and coworkers have verified that cells expressing exclusively Stat5β show a delayed and reduced activation of these two genes (Azam *et al.*, 1995). Previous studies on the C-terminally truncated isoforms of Stat1 and Stat3 have established the precedent that truncated Stats are generated by alternative splicing (Muller *et al.*, 1993; Schaefer *et al.*, 1995; Yan *et al.*, 1995; Caldenhoven *et al.*, 1996). It has been shown that also the Stat5β form can be generated from an alternatively spliced message (the last intron remaining unspliced) (Wang and Yu-Lee, 1996), consistent with transcripts detected in rat liver and mammary glands (Kazansky *et al.*, 1995; Ripperger *et al.*, 1995). More recent studies have suggested that Stat5 truncations can also be generated by proteases; since a nucleus-associated serine protease that cleaves activated Stat5α to generate Stat5β was found to be present in early hematopoietic cells (Azam *et al.*, 1995; Meyer *et al.*, 1998). Recently, Oda and coworkers found that calpain, a ubiquitously expressed cysteine protease, also trims Stat5 within the C-terminus (Oda *et al.*, 2002). The identification of protease activity in several cell lines that are believed to represent earlier stages in myeloid differentiation suggests that the protease activity may be involved in the regulation of lineage-specific Stat5 signaling.

2.1.2. Serine phosphorylation

Tyrosine phosphorylation regulates the dimerization of Stats and is an essential prerequisite for the establishment of the classical Jak/Stat signaling pathway. However, most vertebrate Stats contain a second phosphorylation site within their C-terminus. The phosphorylated residue is a serine contained within a proline-serine-methionine-proline (PSMP) motif, and in the majority of situations its mutation to alanine alters transcriptional activity. Initial studies demonstrated the positive effect of Ser727 phosphorylation on transcriptional activity in Stat1 and Stat3 (Wen *et al.*, 1995; Zhang *et al.*, 1995), but more recent studies indicated that serine phosphorylation enhances also transcriptional activity of Stat4 (Visconti *et al.*, 2000). Also Stat5 and Stat6 are phosphorylated on serine (Kirken *et al.*, 1997; Pesu *et al.*, 2000; Wick and Berton, 2000), but enhanced transcriptional activity has not convincingly been demonstrated. In PRL-stimulated cells, both Stat5A and Stat5B are phosphorylated on a conserved serine residue (Ser725 in Stat5A and Ser730 in Stat5B) located within a PSP sequence, which corresponds in location to the PSMP serine phosphorylation sequence of Stat1, -3, and -4 (Yamashita *et al.*, 1998). Stat5A is additionally phosphorylated on Ser779 (Pircher *et al.*, 1999; Beuvink *et al.*, 2000). The mitogen-activated protein kinase (MAPK) cascade is one candidate that could mediate serine phosphorylation on Stat5 (Pircher *et al.*, 1997; 1999; Yamashita *et al.*, 1998). Serine phosphorylation of Stat5A was not found to enhance Stat5A-mediated signaling in response to IL-2 (Xue *et al.*, 2002), and no difference in PRL-stimulated Stat5 reporter gene activity was seen with serine-to-alanine mutant forms of Stat5B (Ser730Ala) or Stat5A (Ser725Ala, Ser779Ala, or the Ser725,779Ala double mutant) compared with the corresponding wild-type Stat5 forms (Yamashita *et al.*, 1998; Beuvink *et al.*, 2000). In another study, serine phosphorylation has been shown to inhibit Stat5A activation following PRL stimulation. Surprisingly,

costimulation of glucocorticoid receptors completely reversed this suppressive effect of Stat5A serine phosphorylation, and suggested that serine phosphorylation might in some cases limit the activity of Stat5A in the absence of proper coactivation (Yamashita *et al.*, 2001). Delayed tyrosine dephosphorylation and sustained DNA-binding activity were, however, reported for Stat5A-Ser725Ala in cells stimulated with PRL, suggesting that Ser725 phosphorylation has an impact on signal duration (Beuvink *et al.*, 2000).

2.1.3. Dephosphorylation

Shp-2 is a SH2 domain-containing tyrosine phosphatase that is widely expressed in all tissues and appears to be involved in multiple signaling pathways as a positive or a negative regulator (Feng *et al.*, 1994; Neel and Tonks, 1997). Shp-2 has recently been shown to be a phosphatase for Stat5 (Aubert *et al.*, 2003). Shp-2 interacted with Stat5A in a tyrosine phosphorylation-dependent manner and overexpression of Shp-2 impaired EPO-induced tyrosine phosphorylation of Stat5A. In addition, Shp-2 deficiency dramatically delayed dephosphorylation of Stat5A following cytokine removal. Nonetheless, in Shp-2-deficient cells, Stat5A is still dephosphorylated, albeit the process is delayed. It is therefore possible that there is another phosphatase(s) involved in the dephosphorylation of Stat5A. Previous studies have suggested that cytoplasmic protein-tyrosine phosphatase 1B (PTP-1B) negatively regulates the Stat5 activation in PRL-mediated signaling pathway (Aoki and Matsuda, 2000), and that serine/threonine protein phosphatase 2A (PP2A) plays a negative regulatory role in regulating IL-3 signaling by interacting with Stat5 and dephosphorylating it (Yokoyama *et al.*, 2001). Given the fact that these phosphatases are mainly distributed in the cytoplasm, it is reasonable to assume that the dephosphorylation of Stat5A primarily occurs in the cytoplasm. In this regard, it is interesting that a recently identified Stat1 phosphatase, T-cell protein-tyrosine phosphatase TC-PTP, which dephosphorylates Stat1 in both the nucleus and cytoplasm (ten Hoeve *et al.*, 2002), has also been reported to be a potential negative regulator of the PRL-mediated signaling pathway by dephosphorylating Stat5A and Stat5B in the nucleus (Aoki *et al.*, 2002).

2.1.4. Suppressors of cytokine signaling (SOCS)

Currently there are eight known members (SOCS-1-7 and CIS) in the suppressors of cytokine signaling SOCS family, and many of the members have been implicated in the regulation of Stat5 signaling. A central SH2 domain and a unique motif in their C-terminus, which has been designated as a SOCS box, characterize SOCS proteins (Figure 7). Interactions between the SOCS box, elongins B and C, and the ubiquitin-proteasome system appear to influence the rate of SOCS protein degradation (Kamura *et al.*, 1998; Zhang *et al.*, 1999a). The mRNA for SOCS proteins is found at low levels in different cells or tissues (reviewed in Starr *et al.*, 1997; Chen *et al.*, 2000; Krebs and Hilton, 2000; Yasukawa *et al.*, 2000). A wide range of cytokines such as PRL, GH, and ILs induces the expression of SOCS. In most cases, however, there is no correlation between a particular cytokine and the SOCS mRNAs it can induce. Indeed, cytokine induction of *socs* genes often varies with respect to the cell line or tissue. It is now apparent that the Stat proteins contribute significantly to the transcriptional up-regulation of the *socs* genes (reviewed in Chen *et al.*, 2000; Krebs and Hilton, 2000; Yasukawa *et al.*, 2000). Induction of SOCS-1 transcription by IFNγ is mediated by Stat1 (Saito *et al.*, 2000), but binding sites for Stat3 and Stat6 are also found from the SOCS-1 promoter (reviewed in Krebs and Hilton, 2000). The CIS promoter contains four Stat5-binding sites, which are required for the EPO-mediated *cis* gene activation (Matsumoto

et al., 1997). Also a role for Stat5B in the regulation of *socs-2* and *socs-3* gene expression in liver has been suggested (Davey *et al.*, 1999).

Figure 7. The domain structure of SOCS proteins consists of a central SH2 domain and a SOCS box in the C-terminus.

The first member of SOCS family was denoted as CIS, for cytokine-inducible SH2-containing protein (Yoshimura *et al.*, 1995; Masuhara *et al.*, 1997). CIS was cloned originally as an immediate early gene that was induced by IL-2, IL-3, and EPO (Matsumoto *et al.*, 1997). Forced expression of CIS could partially suppress IL-3- or EPO-induced proliferation as well as Stat5 activation (Yoshimura *et al.*, 1995; Matsumoto *et al.*, 1997). CIS acts as a negative feedback regulator of the Jak-Stat5 pathway. The negative effects of CIS on Stat5 activity were confirmed by the phenotypes observed in CIS-transgenic mice (Matsumoto *et al.*, 1999) (Table 4). CIS inhibits signaling by interacting with activated cytokine receptors. At the EPO receptor, CIS binds specifically to the phosphorylated tyrosine residue Tyr401, which is one of the major Stat5 binding sites (Gobert *et al.*, 1996; Klingmuller *et al.*, 1997; Matsumoto *et al.*, 1997). A likely explanation for the inhibition of Stat5 activity would therefore be that Stat5 and CIS compete for binding to the same tyrosine residue at the EPO receptor. In addition, CIS has been shown to bind to the tyrosine phosphorylated IL-3 receptor (Yoshimura *et al.*, 1995) and IL-2 receptor (Aman *et al.*, 1999).

SOCS-1 was identified both as a Jak2-binding protein and as an inhibitor of IL-6 signaling (Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997). SOCS-1 has been shown to interact with all Jak kinases and inhibit signaling by IL-2, IL-3, IL-4, IL-6, GH, LIF, PRL, IFNγ, and EPO (Table 4). The inhibitory mechanism of SOCS-1 has been proposed to involve, in the context of Jak2, a direct interaction with the kinase activation loop, thereby resulting in decreased catalytic activity of Jak2 (Endo *et al.*, 1997; Yasukawa *et al.*, 1999). SOCS-1 is strongly induced by IL-2, can associate with IL-2 receptor, and potently inhibits IL-2-induced Stat5 function, all of which suggest a significant role for SOCS-1 in regulating T-cell immune responses (Sporri *et al.*, 2001).

SOCS-2 has been shown to interact with the GH receptor and to inhibit GH-mediated Stat5 signaling (Ram and Waxman, 1999) (Table 4). SOCS-2 also interacts with the IGF-1 receptor (Dey *et al.*, 1998). SOCS-2 appears to mediate its effects by interaction with the receptor tyrosines (Ram and Waxman, 1999). Knockout and other studies suggested an important role for SOCS-2 in the regulation of growth, possibly by modulating GH and IGF-1 signaling (Greenhalgh *et al.*, 2002).

The expression of SOCS-3 can be induced by many cytokines, including IL-2, IL-3, and EPO (Endo *et al.*, 1997; Naka *et al.*, 1997; Cohney *et al.*, 1999), and by GH, leptin and insulin (Adams *et al.*, 1998; Bjorbaek *et al.*, 1998; Emanuelli *et al.*, 2000) (Table 4). Analyses of either SOCS-3 transgenic or SOCS-3-deficient mice indicated that SOCS-3 plays a critical role in negatively regulating fetal liver erythropoiesis (Marine *et al.*, 1999). Consistent with this, SOCS-3 transgenic mice die of anemia secondary to a block in Epo-Stat5 signaling (Marine *et al.*, 1999). SOCS-3 inhibits also PRL-induced Stat5 activation (Helman *et al.*, 1998). In addition, expression of SOCS-3 is rapidly induced in T-cells in response to IL-2, and SOCS-3 can strongly inhibit IL-2-induced Stat5 phosphorylation (Cohney *et al.*, 1999). Unlike other SOCS proteins, SOCS-3 is rapidly tyrosine phosphorylated after IL-2 stimulation (Cohney *et al.*, 1999). It has been suggested that the phosphorylated SOCS-3 can interact with RasGAP, and also that the phosphorylation can inhibit the SOCS-3-elongin C interaction and activate proteasome-mediated SOCS-3 degradation (Cacalano *et al.*, 2001;

Haan *et al.*, 2003). SOCS-3 appears to function by interacting with Jak1 and inhibiting Jak1 activation (Cohney *et al.*, 1999), but SOCS-3 also binds to Jak2 and activated cytokine receptors (Chen *et al.*, 2000; Sasaki *et al.*, 2000).

NAME	INDUCED BY	ASSOCIATES WITH	INHIBITS SIGNALING BY	KNOCKOUT PHENOTYPES
CIS	IL-1–IL-3, IL-6, IL-12, IL-13, LIF, G-CSF, GM-CSF, TPO, IFNγ, GH, leptin, EPO, PRL, LPS, TSLP, CNTF	EPOR, IL-3R, GHR, IL-2R, PKCθ	IL-2, IL-3, PRL, EPO, IGF-1, GH	No phenotype of null, but enhanced T-cell signaling in transgenic mice
SOCS-1	IL-2, IL-3, IL-6, IL-13, EPO, GH, PRL, GM-CSF, CNTF, thyrotropin	Jak1, Jak2, Jak3, Grb2, Vav, FGFR, PYK2, GHR, c-Kit, Flt3, IGF-1R	IL-2, IL-3, IL-4, IL-6, GH, PRL, EPO, LIF, IFN-γ, IFN-α, OSM, TSLP, TPO, IGF-1	Perinatal lethality owing to unopposed IFNγ-induced liver degeneration
SOCS-2	IL-1–IL-4, IL-6, G-CSF, GM-CSF, EPO, IL-9, GH, PRL, IFNγ, CNTF, IL-10	IGF-1R, PRLR, GHR	GH, IL-6, LIF, IGF-1, PRL	Gigantism owing to unopposed signalling by GH and IGF-1
SOCS-3	IL-1–IL-4, IL-1β, IL-6, IL-7, IL-9-IL-13, M-CSF, G-CSF, EPO, TPO, TNFα, LIF, GH, leptin, PRL, GM-CSF, LPS, insulin, CNTF, thyrotropin	Jak1, Lck, FGFR, Pyk2, GHR, EPOR, leptin receptor, gp130, IGF-1R	IL-2, IL-3, IL-4, IL-6, IL-9, IL-11, GH, PRL, EPO, LIF, IFN-γ, IFN-α, CNTF, leptin, OSM, IGF-1, insulin	Embryonic lethality owing to multiple placental and hematopoietic defects
SOCS-4	?	?	?	?
SOCS-5	?	?	IL-6	?
SOCS-6	?	IRS-1, IRS-4, p85 regulatory subunit of PI3K, InsR	insulin	Growth retardation owing to a requirement for SOCS-6 in the proper regulation downstream of IRS
SOCS-7	?	Ash, Nck, PLCγ	?	?

Table 4. SOCS proteins in cytokine signaling, modified from Krebs and Hilton, 2001; Cooney, 2002; Levy and Darnell, 2002.

2.1.5. Targeted degradation

Ubiquitin-proteosome-dependent degradation plays an important role in the down-regulation of cytokine signaling. Stat proteins typically exhibit long half-lives (Haspel *et al.*, 1996), but the stability of Stat1, Stat2 and Stat3 can be dramatically reduced by infection of the cells with certain negative-strand RNA viruses (*Paramyxovirinae*) (Didcock *et al.*, 1999; Parisien *et al.*, 2001; 2002; Ulane *et al.*, 2003). Proteosome inhibitors have been shown to stabilize the tyrosine phosphorylated Stat5. In addition, the C-terminus of Stat5 has been found to promote degradation (Wang *et al.*, 2000a), suggesting that also the stability of Stat5 might be regulated by degradation. An adapter protein c-Cbl was recently found to negatively regulate GH-stimulated Stat5-mediated transcriptional activation (Goh *et al.*, 2002). In line with this, other studies have also demonstrated that murine embryonic fibroblasts derived from c-Cbl-deficient mice exhibit significantly increased levels of Stat5 protein (Blesofsky *et al.*, 2001). Interestingly, c-Cbl inhibition of GH-stimulated Stat5-mediated transcription was not mediated by interference with events leading up to DNA-binding, but c-Cbl overexpression resulted in increased ubiquitylation and proteosomal degradation of the

tyrosine phosphorylated Stat5. This negative regulation is dependent on the integrity of both the tyrosine kinase-binding domain and the RING finger domain of c-Cbl (Lill *et al.*, 2000; Ota *et al.*, 2000). The c-Cbl RING finger has intrinsic E3 ligase activity that can independently recruit ubiquitin-conjugating enzymes and direct ubiquitin transfer to substrates (Joazeiro *et al.*, 1999; Levkowitz *et al.*, 1999; Yokouchi *et al.*, 1999).

2.1.6. Stat inhibitor proteins

The PIAS (protein inhibitor of activated Stats) family includes five members, PIAS1, PIAS3, PIASy, PIASxα, and PIASxβ (reviewed in Shuai, 2000). PIAS proteins share several structural elements, which are well conserved along all family members. It was recently predicted that PIAS proteins harbor a putative DNA-binding domain, the SAP (SAF-A, Acinus, PIAS) module, at their N-terminus (Kipp *et al.*, 2000). In addition, PIAS3 has a putative RING finger-like domain and the C-terminus of PIAS3 contains an acidic region (Jimenez-Lara *et al.*, 2002). This same C-terminal region of PIAS1 has been shown to be involved in the interaction with Stat1 and required for the inhibition of Stat1-dependent gene activation (Liao *et al.*, 2000). PIAS proteins can bind to distinct classes of nuclear proteins and enhance or repress the transcriptional activities of structurally unrelated factors, such as steroid receptors and Stats (Chung *et al.*, 1997; Liu *et al.*, 1998; Moilanen *et al.*, 1999; Tan *et al.*, 2000). PIAS1 binds to activated Stat1 dimers, thereby blocking their ability to bind DNA (Liao *et al.*, 2000). Analogously, PIAS3 blocks Stat3 DNA-binding activity and Stat3-mediated gene activation (Chung *et al.*, 1997). PIAS3 was recently shown to inhibit also Stat5 (Rycyzyn and Clevenger, 2002). PIAS proteins were shown to function as SUMO-1 (small ubiquitin-related modifier 1) -tethering proteins and zinc finger-dependent E3 SUMO protein ligases, and these properties possibly explain their ability to modulate the activities of various transcription factors (Kotaja *et al.*, 2002). Sumoylation has been shown to be involved in the regulation of subcellular localization, stability, and activity of a variety of proteins (Chauchereau *et al.*, 2003).

2.2. Nuclear translocation of Stat5

In unstimulated cells, Stats predominantly localize to the cytoplasm. Upon stimulation, Stats translocate rapidly to the nucleus and induce gene expression. After termination of the signal, Stats translocate back to the cytoplasm. Like all molecules larger than ~60 kDa, Stats are transported across the nuclear pore complex (NPC) in an active bidirectional process that is energy- and activation-dependent (Doye and Hurt, 1997). Subsequent studies have determined that the hydrolysis of GTP by Ran, a Ras-like small GTPase, provides energy required for the IFNγ-dependent nuclear import of Stat1 (Sekimoto *et al.*, 1996). Active nuclear import of proteins is directed by a short amino acid sequence called a nuclear localization signal (NLS). The NLS is recognized and bound by members of the nuclear receptor family proteins, called importins. Importin-α recognizes the NLS and then functions as an adapter by binding importin-β (Gorlich *et al.*, 1994). Importin-β in turn interacts with NPC and mediates the transport of the cargo into the nucleus. The rapid ligand-mediated nuclear import of Stats has been shown to be dependent on tyrosine phosphorylation but not on association with the cytoskeleton (Lillemeier *et al.*, 2001). To date, no classical NLS has been detected in Stats. Studies have noted a potential role of the DNA-binding domain of Stat in nuclear accumulation and suggested that a cluster of basic residues in DNA-binding domain may have an import function (Herrington *et al.*, 1999; Melen *et al.*, 2001). The identification of putative NLS elements in cytokine receptors and ligands led to the

speculation that Stats may translocate to the nucleus through association with other signaling components (Subramaniam *et al.*, 2000).

The presence of Stats in the nucleus is transient and during the subsequent period of signal decay they are re-exported back to the cytoplasm in preparation for the next round of signaling. Analogous to import, nuclear export is specified by nuclear export signals (NES) (Fornerod *et al.*, 1997). The export receptor CRM1 interacts with the NES in a Ran-GTP-dependent manner and transports the protein back to the cytoplasm (Fornerod *et al.*, 1997). Recent studies on Stat1 have provided important insights into how this protein is exported from the nucleus (Begitt *et al.*, 2000; McBride *et al.*, 2000; Mowen and David, 2000). Stat1-NES, which is a leucine-rich helical segment present in the coiled-coil domain, appears to be masked when the dimers are bound to DNA (Begitt *et al.*, 2000). During the period of signal decay, Stat1 is dephosphorylated leading to its dissociation from DNA, after which the NES becomes accessible to the CRM1 export carrier.

The nuclear sojourn time of Stat5 is about 6 min, as shown by Swameye and coworkers (Swameye *et al.*, 2003). Extrapolating from studies of other Stats, it can be assumed that the tyrosine phosphorylation and dimer formation are prerequisite to the translocation of Stat5 homo- and heterodimers to the nuclear compartment (Heim *et al.*, 1995; Sasse *et al.*, 1997; Mowen and David, 1998; Herrington *et al.*, 1999). Consistent with this model, tyrosine phosphorylation of Stat5A and Stat5B has been demonstrated to be critical for Stat5 DNA-binding and transcriptional activation (Gouilleux *et al.*, 1994; Barahmand-Pour *et al.*, 1998; Herrington *et al.*, 1999). Although tyrosine phosphorylation and dimerization precede nuclear translocation, a study of PRL receptor mutants suggested that Stat5 tyrosine phosphorylation and nuclear translocation are separately regulated events (Ali, 1998). Another study suggested that the DNA-binding activity of Stat5 was required for the GH-induced nuclear accumulation of Stat5 (Herrington *et al.*, 1999). No *bona fide* nuclear import signal has been identified in Stat5 (Herrington *et al.*, 1999), but the dimerization-induced unmasking of a cryptic import signal in Stats has been suggested (Milocco *et al.*, 1999). The export mechanism from the nucleus for Stat5 is unknown, but probably it is similar to the mechanism presented for Stat1.

2.3. Stat5 in transcriptional activation

2.3.1. Interaction of Stat5 with other transcription factors and coregulators

The transcriptional regulation of eukaryotic genes involves the specific and ordered interaction of a large number of proteins including enhancer/promoter-specific transcription factors, chromosomal remodeling complexes, and components of the basal transcriptional machinery (reviewed in Blackwood and Kadonaga, 1998; von Hippel, 1998). Gene regulation is therefore a highly coordinated effort, which helps to ensure that correct genes are expressed only under appropriate circumstances. The first evidence of an interaction between Stats and other transcription factors came from the copurification of IRF-9 with Stat1 and Stat2 in the ISGF-3 complex (Eilers *et al.*, 1993). Subsequent studies of Stat-dependent promoters provided evidence for the interaction with other transcription factors. One group of Stat-binding coactivators are histone acetyltransferases (HATs), especially CBP/p300 (Paulson *et al.*, 1999), but Stats bind also with other coregulator proteins. The molecular mechanisms that underlie Stat5-mediated transcription are not fully understood, but these mechanisms involve interactions and cooperation with both sequence-specific transcription factors as well as with transcriptional coregulators. The proteins shown to interact with Stat5 include glucocorticoid receptor (GR), centrosomal P4.1-associated protein (CPAP) (Peng *et al.*, 2002), N-Myc interactor (Nmi) (Zhu *et al.*, 1999), Specificity protein (Sp)1 (Martino *et al.*, 2001), Ying Yang-1 (YY1) (Bergad *et al.*, 2000), and C/EBPβ (Wyszomierski and Rosen, 2001). Stat5

interacts also with negative regulators of transcription, such as nuclear receptor corepressor, silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT) (Nakajima *et al.*, 2001).

2.3.1.1. Stat5 in transcriptional control in PRL signaling

The main actions of PRL, a polypeptide hormone secreted mainly by the pituitary and also by peripheral tissues, are related to lactation and reproduction. The biological effects of PRL can, however, be subdivided into five broad categories: reproduction, osmoregulation, growth, integument, and synergism with steroids (reviewed in Bole-Feysot *et al*., 1998). The PRL actions result from the interaction of PRL with its receptor, which leads to the activation of a cascade of intracellular events (reviewed in Groner and Gouilleux, 1995; Hynes *et al.*, 1997). Although the Jak2-Stat5 cascade is probably the most important signaling pathway used by PRLR, other pathways are also involved. Binding of Stat5 to the gene promoter is necessary for the induction of the expression of β-casein, αs1-casein (Jolivet *et al.*, 1996), whey acidic protein (Li and Rosen, 1995), and β-lactoglobulin milk proteins (Burdon *et al.*, 1994). The physiological function of Stat5 in PRL signaling is clearly demonstrated in the knockout models, where Stat5-null mice show a mammary phenotype similar to that of the PRLR-deficient mice, exhibiting impaired differentiation of lobuloalveolar units and inability to lactate (Liu *et al.*, 1997; Ormandy *et al.*, 1997a; 1997b; Teglund *et al.*, 1998; Miyoshi *et al.*, 2001).

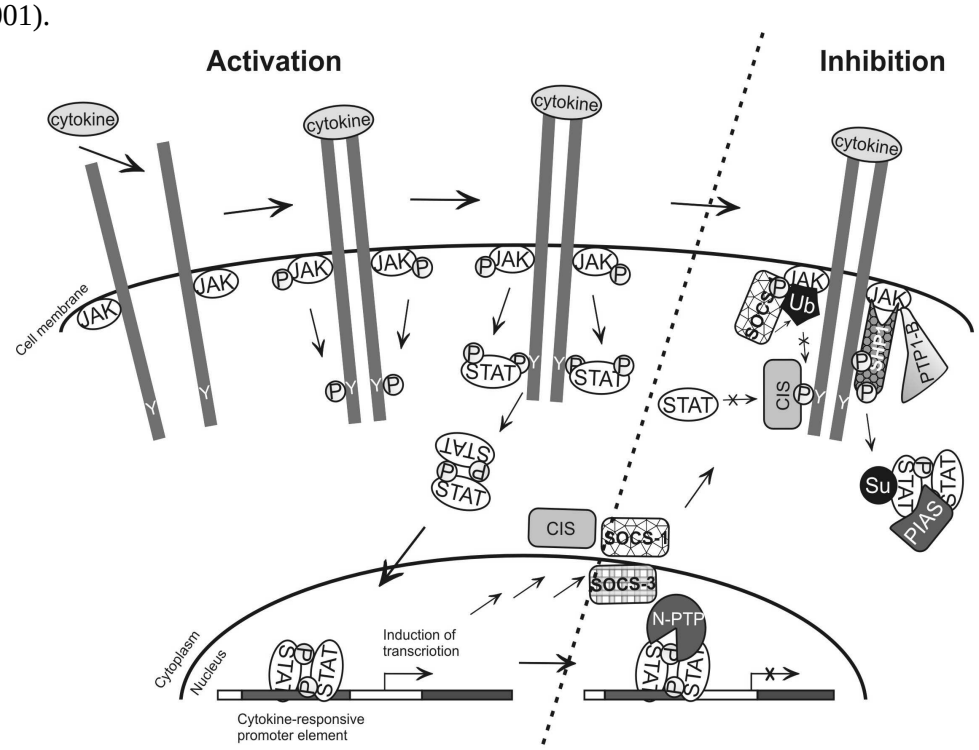

Figure 8. Model for the hormonal synergism between PRL and glucocorticoids in the induction of the β-casein gene. Stat5 is activated after PRL binding to its receptor. Dexamethasone (Dex) binds to GR in the cytoplasm. Stat5 dimer and GR-Dex form then a complex, which translocates into the nucleus, binds to the β-casein promoter, and initiates transcription. In contrast to the requirement for a specific Stat5-binding site at the β-casein promoter, no functional GRE is necessary. The synergistic activation of transcription needs Stat5 activation and the presence of the transactivation function in the GR. Modified from Groner, 2002.

The hormonal synergism between PRL and glucocorticoids in the induction of the β-casein gene has been investigated in molecular detail (Doppler *et al.*, 1989; 1990; 1995; Lechner *et al.*, 1997b) (Figure 8). When glucocorticoid hormones are taken up into cells, they bind intracellularly to the latent form of the GR. Upon ligand binding, GR, which otherwise resides in the cytoplasm, translocates to the nucleus where it binds to palindromic glucocorticoid response elements (GREs) in the promoters of target genes (Beato *et al.*, 1995; Bamberger *et al.*, 1996). GR can also interact with GRE half-sites, a DNA-interaction that can be stabilized by other transacting factors binding to adjacent sequences. A single Stat5-binding site in the promoter region of a target gene is sufficient to confer the functional interaction, but the synergism is enhanced when multimerized Stat5-binding sites are present. The synergistic activation of transcription needs Stat5 activation and the presence of the transactivation function in the GR. In contrast to the requirement for a specific Stat5-binding site, no functional GRE is necessary (Stoecklin *et al.*, 1996; 1997; 1999). Recently, Doppler and coworkers reported that GR enhances Stat5 activity in the absence of a functional DNA-binding domain and GR-binding sites only when high expression levels are used, suggesting that the mode of action of the GR may depend on the concentration of the GR (Doppler *et al.*, 2001). The regulatory factors governing milk protein gene expression are not limited to Stat5 and the GR, but include additional signal-regulated components (Figures 9 and 10). These include C/EBPs, NF1, YY1 (Stocklin *et al.*, 1996; 1997; Robinson *et al.*, 1998; Wyszomierski and Rosen, 2001) and other transcription factors characterized in less detail (Altiok and Groner, 1994; Rosen *et al.*, 1998; 1999). In addition, Stat5 associates with CBP/p300 at the β-casein promoter (Pfitzner *et al.*, 1998).

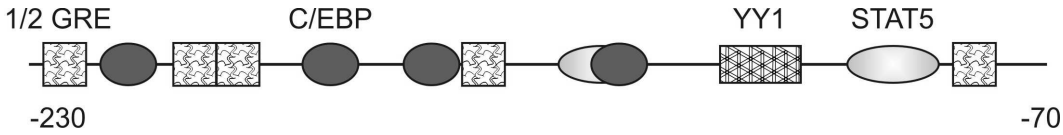

Figure 9. Diagrammatic representation of the rat β-casein promoter including the response elements for critical transcription factors, adapted from Rosen *et al.*, 1999.

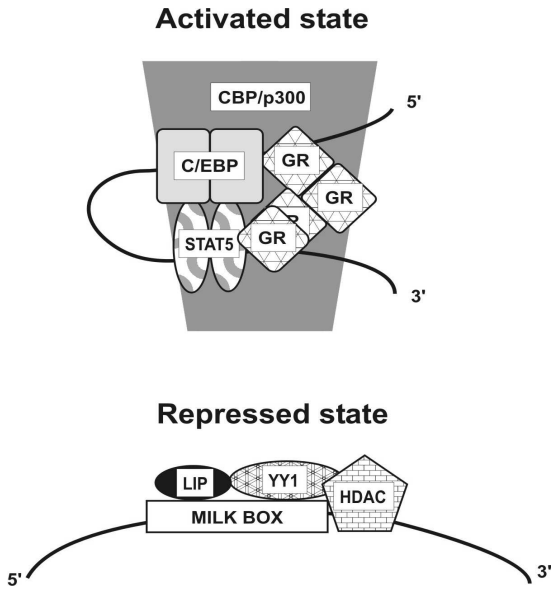

Figure 10. Model for protein interactions at the β-casein promoter involved in chromatin remodeling. In the active state, interactions between Stat5, C/EBPβ, and GR create a stable activation complex. The three proteins together recruit CBP/p300 to the β-casein promoter, causing histone acetylation and a high level of transcriptional activation. Also the proteins involved in the repressed state have been characterized. In the repressed state, the LIP (liver-enriched inhibitory protein), which is an inhibitory form of C/EBPβ, may tie YY1 to the weak YY1-binding site at the β-casein promoter. YY1 may then recruit histone deacetylases (HDACs), resulting in histone deacetylation and repression of transcription. Adapted from Rosen *et al.*, 1999.

2.3.2. Genes regulated by Stat5

In the nucleus, dimerized Stat5 interacts directly with specific DNA elements through a centrally located DNA-binding domain. Studies on Stat5-responsive gene promoters have shown that Stat5 recognizes and binds to canonical GAS sites with the general sequence TTCNNNGA (reviewed in Ihle, 1996). Because multiple Stats retain ability to bind the same highly conserved sequences, there is significant overlap in the DNA site recognition between Stat5, Stat1, and Stat3 (reviewed in Decker *et al.*, 1997; Leonard and O'Shea, 1998). The functions of individual Stat proteins are often redundant due to overlapping specificities for common promoter elements. Stat dimers can undergo tandem linkage through their N-terminal domains when bound to closely located GAS sites (Xu *et al.*, 1996a; Verdier *et al.*, 1998). Stabilization achieved by these complexes can thus lead to transcriptional activation from coupled GAS sites that individually are too weak to drive transcription (Verdier *et al.*, 1998; John *et al.*, 1999). Tandem Stat5-response elements have been found in the promoter of the IL-2 receptor α (Verdier *et al.*, 1998; John *et al.*, 1999), and also in the promoters of the CIS and the Serine protease 2.1 (Spi2.1) (Bergad *et al.*, 1995; Minamoto *et al.*, 1997).

Stat5 regulates immediate early cytokine-inducible genes (Table 4). These targets include *pim-1*, *osm*, and *cis* in hematopoietic cells (Lilly *et al.*, 1992; Yoshimura and Arai, 1996; Matsumoto *et al.*, 1997), and genes responsive to pulsatile action of GH in the male rat liver (Gebert *et al.*, 1997; Udy *et al.*, 1997). In fact, many of the genes, which respond to Stat5 and include defined Stat response elements, are primarily associated with mammary gland milk production and the cell cycle progression of hematopoietic cells. The Stat5-dependent mechanisms involved in protection from apoptosis, growth factor-independent proliferation, and transformation are not known, but the possible role of genes transcriptionally regulated by Stat5, such as *a1* (Lin *et al.*, 1996), *bcl-xL* (Socolovsky *et al.*, 1999), *pim-1* (Lilly *et al.*, 1992), and *osm* (Yoshimura and Arai, 1996) should be considered. A1 and Bcl-xL belong to the Bcl-2 family of proteins that protect cells from apoptosis (Motoyama *et al.*, 1995; Hamasaki *et al.*, 1998). In addition, the human homologue of A1 (Bfl-1) cooperates with the *E1A* oncogene in transformation (D'Sa-Eipper *et al.*, 1996). Activation of *pim-1* gene correlates with mitogenesis and growth factor-independent survival (Lilly *et al.*, 1992; Mochizuki *et al.*, 1999) and OSM is able to stimulate growth of various cells (Gomez-Lechon, 1999).

STAT5-INDUCED GENES
β-casein, β-lactoglobulin, bcl-xL, bile acid cotransporter, carboxyl ester lipase, cyclin D1, cyp19, cyp3a10, insulin, IRF-1, osm, $p21^{waf1/Cip1}$, pim-1, α2-macroglobulin
STAT5-REGULATED GENES WITH TANDEM GAS SITES
il-2 receptor α-chain, spi2.1 and spi2.2, cis, fc receptor γ-chain

Table 4. Examples of Stat5-induced genes, modified from Grimley *et al.*, 1999.

2.3.2.1. Pim-1

The cytoplasmic serine/threonine kinase Pim-1 acts as a survival factor to inhibit apoptosis in myeloid cells deprived of cytokines (Lilly and Kraft, 1997). Pim kinase is a labile protein whose expression is especially high in hematopoietic and germ cells. A single Pim-1 protein of 33 kDa exists in humans, while mice have 33 and 44 kDa forms. The latter results from an alternate translational start site missing in the human gene (Saris *et al.*, 1991). Approximately equal amounts of the two murine forms are expressed in hematopoietic cells (Saris *et al.*, 1991; Lilly and Kraft, 1997). The cloning of the highly homologous *pim-2* (van der Lugt *et al.*, 1995) and *pim-3* genes (Konietzko *et al.*, 1999) suggested that there is a

family of Pim kinases. The expression of Pim-1 is induced by hematopoietic growth factors including IL-2, IL-3, GM-CSF, EPO, but is also induced by PRL (Dautry *et al.*, 1988; Lilly *et al.*, 1992; Miura *et al.*, 1994; Buckley *et al.*, 1995).

The proto-oncogene *pim-1* was originally identified as a preferential integration site of Moloney murine leukemia virus, which induces T-lymphomas in mice (Cuypers *et al.*, 1984). Oncogenity of Pim-1 has been characterized in both transgenic and retroviral models (Selten *et al.*, 1986; Breuer *et al.*, 1989). Accordingly, overexpression of Pim-1 protein is seen in erythroleukemias (Dreyfus *et al.*, 1990), various human leukemias (Amson *et al.*, 1989), and in B-cell lymphomas (Verbeek *et al.*, 1991). By itself, Pim-1 has low oncogenic potential, but it strongly cooperates with c-Myc, L-Myc, N-Myc, Bcl-2, and Gfi-1 in T-cell lymphomagenesis (van Lohuizen *et al.*, 1989; Moroy *et al.*, 1991; Schmidt *et al.*, 1998; Wang *et al.*, 2001b). Pim-1 has been shown to be involved in the IL-3 signal transduction pathway, in cell cycle regulation and proliferation, and in apoptosis and cell survival (reviewed in Wang *et al.*, 2001b).

Some substrates for Pim-1 have been identified; p100 (originally identified as a coactivator for EBNA-2) (Leverson *et al.*, 1998), a cell cycle phosphatase cdc25A (Mochizuki *et al.*, 1999), heterochromatin protein 1 (HP1) (Koike *et al.*, 2000), Pim-1-associated protein 1 (PAP1) (Maita *et al.*, 2000), a tyrosine phosphatase PTP-U2S (Wang *et al.*, 2001a), NFATc1 transcription factor (Rainio *et al.*, 2002), tumor necrosis factor receptor-associated factor 4-associated factor 2/sorting nexin 6 (TFAF2/SNX6) (Ishibashi *et al.*, 2001), nuclear mitotic apparatus protein (NuMA) (Bhattacharya *et al.*, 2002), and a cyclin-dependent kinase inhibitor p21 (Wang *et al.*, 2002). Recently, Pim-1 and Pim-2 were shown to regulate the stability of SOCS-1 by phosphorylating it (Chen *et al.*, 2002).

AIMS OF THE PRESENT STUDY

Stat5 mediates signals for a variety of cytokines and growth factors. Stat5 activation is often an integral component of redundant signal cascades involving complex cross-talk. However, relatively little is known about how Stat5 activation is regulated in the cell. The transcriptional coregulators that interact with Stat5 and function together with Stat5 to regulate gene transcription are still largely unknown. Also the exact mechanisms by which Stat5 signal is down-regulated after specific stimulation have remained somewhat elusive.

The detailed aims of this thesis study were:

1) To study the activation of Jak kinases and Stat factors upon PDGF stimulation.

2) To characterize coregulators for the Stat5-mediated transcription.

3) To study the effect of a Stat5 target gene Pim-1 on Stat5 activity.

MATERIALS AND METHODS

1. Cell lines and stimulations

Cell lines

Name and description	**Used in**	**Culture medium** **(all mammalian cell culture medias include glutamin, penicillin, and streptomycin)**
293T, Human kidney epithelial cells, ATCC	II, III	D-MEM+10%FBS
COS-7, African green monkey kidney epithelial cells, SV40 transformed, ATCC	I, II, III, IV	D-MEM+10%FBS
Swiss 3T3, mouse fibroblasts, ATCC	I	D-MEM+10%FBS
NIH 3T3, normal mouse fibroblasts, ATCC	I	D-MEM+10%NBCS
Src$^{-/-}$ and Fyn$^{-/-}$ mouse fibroblasts, derived from Src$^{-/-}$ and Fyn$^{-/-}$ mice (Soriano *et al.*, 1991; Stein *et al.*, 1992), were gift from Dr. P. Soriano.	II	D-MEM+10%NBCS
32D, ATCC	I	RPMI 1640+10%FBS + 2.5 ng/ml mIL-3
PAE cells expressing wt PDGF α-R (Eriksson *et al.*, 1992b), **wt β-R**, or **β-R mutants Y579F, Y581F** (Mori *et al.*, 1993)	I, II	Ham's F-12 medium+10%FBS
PAE cells expressing PDGF β-R mutants K634A (Sorkin *et al.*, 1991); **Y716F** (Arvidsson *et al.*, 1994); **Y740/751F** (Wennstrom *et al.*, 1994); **Y1009/1021F** (Ronnstrand *et al.*, 1992); **Y771F, Y775F, Y778F** (Ruusala *et al.*, 1998).	I	Ham's F-12 medium+10%FBS
Sf-9, insect cells (Smith *et al.*, 1985)	II	Sf900 medium (Invitrogen)+10%FBS
FDCP1/neo and **FDCP1/Pim**, (Lilly and Kraft, 1997)	IV	RPMI 1640+10%FBS + 2ng/ml mIL-3 or 10% WEHI medium
HC11 (Ball *et al.*, 1988)	III, IV	RPMI 1640+10%FBS+5µg/ml insulin+10ng/ml EGF + 50µg/ml Gentamycin
HC11-pCIneo, HC11-p100	III	RPMI 1640+10%FBS+5µg/ml insulin+10ng/ml EGF +geneticin (G418, Life Technologies)
HeLa, ATCC	IV	MEM+10%FBS

Cytokines, growth factors and hormones

Cytokine, growth factor, hormone	Used in	Company
PDGF-AA	I	Upstate Biotechnology
PDGF-BB	I, II	Upstate Biotechnology
Prolactin (PRL)	III, IV	Sigma-Aldrich
Dexamethasone (Dex)	III	Sigma-Aldrich
Epidermal growth factor (EGF)	III, IV	Sigma-Aldrich
Insulin	III, IV	Sigma-Aldrich
Interleukin 3 (IL-3)	IV	Peprotech
Erythropoietin (EPO)	IV	Eprex; Janssen-Cilag

Stimulations

Before stimulation COS-7 and 293T cells were starved overnight without serum, followed by stimulation with 50 ng/ml PDGF-BB for 10 min at 37 ºC. All fibroblasts were stimulated with 100 ng/ml PDGF-BB for 10 min at 37 ºC after overnight starvation in 0.2% NBCS. PAE cells were stimulated with 100 ng/ml PDGF-BB for 10 min at 37 ºC. 32D cells were starved without IL-3 and serum overnight and stimulated with 10 ng/ml mIL-3 for 15 min. Prior to treatment with lactogenic hormones, confluent HC11 cells were starved for 48 h in medium lacking EGF, but containing 5 µg/ml insulin and 2% FBS. Stimulation was done in starvation media with 5 µg/ml PRL and 100 nM Dex for 15 min - 20 h. FDCP cells were starved for 6 h, after which they were stimulated with 10 ng/ml mIL-3 for 16 h. HeLa cells transfected with EPO receptor were starved without serum and stimulated with 10 U/ml EPO for 6 h.

2. Baculoviruses

Virus	Used in	Reference/Source
βRic	II	(Hansen *et al.*, 1996)
Jak1	II	Dr. J. Ihle (Quelle *et al.*, 1995)
c-Src	II	Dr. D. O. Morgan
Stat5A	II	Dr. T. Wood

3. Methods used

Method	Used and described in
Cell lysis	I, II, III, IV
Electrophoretic mobility shift assay (EMSA)	I, II, III, IV
GST pulldown	III, IV
Immunoprecipitation	I, II, III, IV
In vitro kinase assay	I, II, IV
Luciferase assay	III, IV
Nonradioactive kinase assay	II
Peptide binding/peptide inhibition	I
PRE binding	I
Transfection	I, II, III, IV
Western blotting	I, II, III, IV

4. DNA constructs

DNA constructs	Used in	Reference/Source
pSV7d	I, II	(Truett *et al.*, 1985)
PDGF β-R-pSV7d	I, II	(Claesson-Welsh *et al.*, 1988)
PDGF β-R-pCIneo	II	II
PDGF β-R mutants Tyr579Phe (Y579F)- and Y581F-pSV7d	I	(Mori *et al.*, 1993)
PDGF β-R mutants Y579F-pCIneo and Y581F-pCIneo	II	II
pCIneo	II, III, IV	Promega
Jak1	II	(Silvennoinen *et al.*, 1993a)
c-Src	II	(Silvennoinen *et al.*, 1993b) and II
Src-KN (Lys297Met)	II	Dr. D. O. Morgan
sheep Stat5A (MGF)	I, II	Dr. B. Groner (Wakao *et al.*, 1994)
Mouse Stat5A	II, III, IV	Dr. T. Wood
SH2 mutant (Arg618Leu) of mouse Stat5A	II, III	Dr. T. Wood
pSpi-luc2	III, IV	Dr. T. Wood (Sliva *et al.*, 1994)
Jak1-KN (mutation Lys835Glu)	II	(Silvennoinen *et al.*, 1993a) and II
Jak2-KN-HA (mutation Lys882Glu)	II	(Saharinen *et al.*, 1997) and II
pSG5-p100-Flag	III, IV	(Yang *et al.*, 2002)
pSG5-SN-Flag (amino acids 1-639)	III	(Yang *et al.*, 2002)
pSG5-TD-Flag (amino acids 640-885)	III	(Yang *et al.*, 2002)
SN-GST and TD-GST	III	(Yang *et al.*, 2002)
pGEX-4T-1	II, III, IV	Amersham Pharmacia Biotech
Stat5A-TAD-GST	III, IV	III
pZZ1	III	(Gouilleux *et al.*, 1994)
C/EBPβ luciferase reporter	III	(Pesu *et al.*, 2000)
NF-κB luciferase reporter	III	(Saksela and Baltimore, 1993)
CBP-HA	III	(Chrivia *et al.*, 1993)
pLTR-Pim-1, pLTR-Pim-1(Lys67Met), pECFP-Pim-1	IV	(Rainio *et al.*, 2002)
Pim-GST / Pim(Lys67Met)-GST	IV	(Rainio *et al.*, 2002)
pCMV-Pim-1	IV	(Leverson *et al.*, 1998)
pRLTK	III, IV	Promega
pSV-ß-galactosidase	IV	Promega
pME18S-SOCS-1,-2, and -3	IV	Dr. D. J. Hilton
SOCS-3-pCIneo	IV	Dr. J. A. Johnston
SOCS-3-GST	IV	Dr. J. A. Johnston
pLTRpoly	IV	(Makela *et al.*, 1992)
pEGFP-C1	IV	Clontech Laboratories
NFAT-LUC	IV	Dr. G. R. Crabtree
AP-1-LUC	IV	Dr. G. R. Crabtree

5. Antibodies

Antibodies	Used in	Company, provider or reference
monoclonal anti-phosphotyrosine (clone 4G10)	II, III, IV	Upstate Biotechnology
monoclonal anti-phosphotyrosine (PY20)	I, II	Transduction Laboratories
monoclonal anti-Jak1	II	Transduction Laboratories
polyclonal anti-PDGF β-R	II	Upstate Biotechnology
monoclonal anti-Stat5A	II, III, IV	Zymed Laboratories
monoclonal anti-Flag M2	III, IV	Sigma-Chemical
polyclonal anti-Stat5B (C-17x)	I, III	Santa Cruz Biotechnology
polyclonal c-Src antibody	II	Santa Cruz Biotechnology
monoclonal anti-HA antibody (clone 16B12)	II, III	Berkeley Antibody Company
monoclonal anti-v-Src (Ab-1)	II	Oncogene Research Products
rabbit antiserum against Jak1	I	(Silvennoinen *et al.*, 1993a)
rabbit antiserum against Jak2	I	(Silvennoinen *et al.*, 1993a)
rabbit antiserum against Tyk2	I	Santa Cruz Biotechnology
rabbit antiserum against Stat5	I	Santa Cruz Biotechnology
monoclonal anti-Stat1 antibody	I	Transduction Laboratories
monoclonal anti-Stat3 antibody	I	Transduction Laboratories
monoclonal anti-Stat4 antibody	I	Transduction Laboratories
monoclonal anti-Stat5 antibody	I	Transduction Laboratories
monoclonal anti-Stat6 antibody	I	Transduction Laboratories
monoclonal anti-Jak1 antibody	I, II	Transduction Laboratories
rabbit antiserum against PDGFR-3	I	(Claesson-Welsh *et al.*, 1988; Welsh *et al.*, 1990)
rabbit antiserum against PDGFR-HL2	I	
rabbit antiserum PDGFR-7	I	(Eriksson *et al.*, 1992a)
rabbit antiserum PDGFR-4	I	Dr. S. Courtneidge
rabbit antiserum against Stat3	I	Dr. X. Cao
rabbit antiserum against green fluorescent protein (GFP)	IV	Clontech Laboratories

RESULTS

1. Activation of Stat family members in PDGF signaling (I, II)

1.1. PDGF-induced activation of Stat family members (I)

At the beginning of this study, quite a wealth of knowledge about cytokine receptors activating Jak kinases and Stat factors existed. However, the activation patterns and the mechanisms by which these proteins are activated by growth factor receptors were largely unknown. Upon stimulation, PDGF receptors were known to activate a number of different signaling molecules (reviewed in Heldin, 1997; 1998), and we got interested in studying whether Stat family members are also activated. For this purpose, we examined the phosphorylation of Stat1 in Swiss 3T3 cells expressing high levels of both PDGF α-R and PDGF β-R. Stimulation led to increased Stat1 tyrosine phosphorylation, which was at its maximum after 10 min stimulation (I, Fig. 1A). Subsequently, also Stat3 and Stat5 were found to be tyrosine phosphorylated (I, Fig. 1B and C), while no phosphorylation of Stat2, Stat4, or Stat6 was detected. In addition, both PDGF α- and β-Rs appeared to induce DNA-binding activity of Stat1, Stat3 and Stat5, as shown in electrophoretic gel-mobility shift assay (EMSA) (I, Fig. 2). To compare the strength of PDGF-induced Stat activation to the activation induced by a cytokine, we analyzed nuclear extracts from PDGF-stimulated Swiss 3T3 cells and from IL-3-stimulated 32D cells by EMSA. It appeared that PDGF-BB and IL-3 similarly stimulated the DNA-binding activity of Stat5 (I, Fig. 3).

1.2. Tyrosine phosphorylation of Stat5 requires kinase activity of the PDGF β-R and involves certain receptor tyrosines (I, II)

Our next step was to determine the required elements of the PDGFR system for Stat activation. We selected Stat5 to further studies, because it was strongly activated by PDGF. We first investigated whether the kinase domain of the PDGF β-R was required for tyrosine phosphorylation of Stat5. For this purpose, we compared the activation of Stat5 in porcine aortic endothelial (PAE) cells expressing wild-type PDGF β-R with cells expressing a mutant receptor with amino acid substitution in the catalytic domain (Lys634Ala) that inactivates the catalytic activity of the receptor (Sorkin *et al.*, 1991). Tyrosine phosphorylation of Stat5 was found to be dependent on the catalytic activity of the PDGF β-R (I, Fig. 4).

In cytokine receptor signaling the Stats are recruited through their SH2 domain to the tyrosine phosphorylated receptor complex and this association is a prerequisite for the C-terminal tyrosine phosphorylation. We wanted to know whether the tyrosine phosphorylation of Stat5 was dependent on association with any of the phosphorylation sites in the PDGF β-R. For this purpose, we used PAE cell lines stably expressing different PDGF β-R mutants. In cells expressing receptor mutant Tyr581Phe (Y581F), tyrosine phosphorylation of Stat5 was found to be considerably decreased (I, Fig. 5 and Table 1). There was also a decrease in tyrosine phosphorylation of Stat5 in the cells expressing Y579F or Y775F mutant β-Rs, whereas other receptor mutants showed tyrosine phosphorylation close to that of the wild-type receptor. Also the DNA-binding activity of Stat5 in PAE cells expressing Y581F, Y579F, and Y775F receptor mutants was reduced (I, Fig. 6). Consistent with the results obtained in PAE cells with stably transfected receptor mutants, PDGF β-R mutants failed to induce the phosphorylation of Stat5 also in COS-7 cells (II, Fig. 3A). However, the Y579F and Y581F mutations did not affect autokinase activity of the PDGF β-R, and both mutant receptors were

also able to phosphorylate Stat5 peptide as efficiently as the wild-type receptor (II, Fig. 3B). Thus, the defect in the activation of Stat5 is not due to the reduced catalytic activity of the mutated receptors towards Stat5. Assays with phosphorylated or unphosphorylated peptides corresponding to Y579F, Y581F or Y775F and their surrounding amino acid residues, revealed that Stat5 bound efficiently to peptides containing phosphorylated Y579F, Y581F or Y775F, but very weakly to the corresponding unphosphorylated peptides (I, Fig. 7). In addition, peptides with phosphorylated Y579 or Y581 competed efficiently with binding of Stat5 to all three peptides. Moreover, a peptide with phosphorylated Y775 competed well with the binding of Stat5 to the same immobilized peptide, but less efficiently with the binding to immobilized peptides containing phosphorylated Y579 or Y581. This *in vitro* binding data is consistent with a direct association between Stat5 and phosphorylated Y579, Y581 and Y775. In addition, we found that only wild-type Stat5, but not the SH2 mutant (Arg618Leu) form, became tyrosine phosphorylated upon PDGF stimulation (II, Fig. 4). These results indicated that PDGF-induced activation of Stat5 requires the SH2 domain of Stat5 and that PDGF β-R mutations interfere with the association of Stat5 to the receptor.

2. PDGF β-R, Jak1 and c-Src in the activation of Stat5 (I, II)

2.1. Interaction between the PDGFRs and Jaks (I)

It was now evident that PDGF receptors activated Stats. The activation mechanism was, however, unknown. In cytokine signaling, Jak kinases are thought to be the predominant activators for Stats and their activation was therefore interesting also in our PDGF-related studies. We first studied the interaction between Jak proteins and the PDGF receptors. PDGF β-R was brought down from ligand-stimulated cells by an antiserum against Jak1, and to a considerably lesser extent by antisera against Jak2 or Tyk2 (I, Figs. 8A and 8B). Jak1 coprecipitated to some extent also with the unstimulated receptor, whereas stimulation resulted in an increased association between Jak1 and the receptor (I, Fig. 9A). We also investigated the ability of the PDGF α-R to associate with different Jaks. PDGF α-R was seen in Jak1 immunoprecipitations after stimulation, but not after immunoprecipitation with antisera against Jak2 or Tyk2 (data not shown). We also saw an induction in the level of tyrosine phosphorylated Jak1 after stimulation (I, Fig. 9B). To estimate the kinase activity of Jak1 in response to PDGF, the immunoprecipitated Jak1 was assayed in *in vitro* kinase assay with a substrate peptide corresponding to the Jak2 phosphorylation site (Silvennoinen *et al.*, 1993c; Feng *et al.*, 1997). PDGF clearly induced the kinase activity of Jak1 as compared to unstimulated cells (I, Fig. 9C). Taken together, these results demonstrate that the PDGF β-receptor associates with Jak1 and to a lesser extent with Jak2 and Tyk2, whereas the activated PDGF α-R associates only weakly with Jak1 and not detectable with Jak2 and Tyk2. The association between Jak1 and the receptor, as well as the tyrosine phosphorylation and kinase activity of Jak1, were increased by PDGF.

2.2. Activation of Stat5 by PDGF β-R, Jak1 and c-Src (II)

Jak kinases are activated upon PDGF stimulation, but so are Src family kinases and the PDGFR itself. To gain insight into the individual roles of different PDGF-induced tyrosine kinases in the activation of Stat5, we analyzed the abilities of PDGF β-R, c-Src, and Jak1 to activate Stat5 in insect and mammalian cells. Jak1 was chosen for these studies, since it appeared in our studies to be the predominantly activated Jak kinase in PDGF β-R signaling. Also Src kinases were interesting, since their activation was shown to lead to Stat5

activation in several occasions (Yu *et al.*, 1995; Cao *et al.*, 1996; Chaturvedi *et al.*, 1997; 1998). Furthermore, Src kinases c-Src, Fyn and c-Yes were known to bind directly to the PDGF β-R autophosphorylation sites Y579 and Y581 (Anderson *et al.*, 1990; Twamley-Stein *et al.*, 1993), which were found to be critical for the activation of Stat5 in our studies. In addition, there were reports about different RTKs to be able to activate Stat proteins directly (Quelle *et al.*, 1995; Park *et al.*, 1996; Chen *et al.*, 1997; Choudhury *et al.*, 1998).

Our results in insect cells showed that Stat5 became efficiently phoshorylated in cells expressing the intracellular part of the PDGF β-R (βRic) and to a lesser extent in Jak1 expressing cells (II, Fig. 1A). Low levels of tyrosine phosphorylation of Stat5 could also be detected in c-Src expressing cells. The DNA-binding activity of Stat5 was analyzed in EMSA, and again the receptor intracellular region and Jak1 clearly induced the DNA-binding activity of Stat5 (II, Fig. 1B). In contrast, the DNA-binding activity of Stat5 was only weakly induced by c-Src. The kinases were found to be tyrosine phosphorylated at similar levels (II, Fig. 1C). However, tyrosine phosphorylation does not always correlate with the kinase activity, and in autokinase assays c-Src showed high kinase activity (II, Fig. 1D). In line with the results from insect cells, PDGF β-R, as well as Jak1, induced DNA-binding activity of Stat5 when coexpressed in 293T cells (II, Fig. 2A). c-Src, which was expressed in mammalian cells, showed a strong autokinase activity (II, Fig. 2B), but was not able to induce the DNA-binding activity of Stat5. The results with immunoprecipitated kinases subjected to *in vitro* peptide kinase assays with Stat5 using peptide containing Y694 as a substrate, showed that PDGF β-R, as well as c-Src, were capable to phosphorylate the Stat5 peptide (II, Fig. 2B). However, taken into account the high autokinase activity of c-Src, the Y694 site did not appear to be an optimal substrate for c-Src. In several autokinase assay experiments Jak1 phosphorylated the peptide at similar level as PDGF β-R.

The efficient phosphorylation of Stat5 by βRic in insect cells suggested that PDGF β-R might be the kinase phosphorylating Stat5 in PDGF signaling. However, we wanted to confirm that the PDGF β-R-induced activation of Stat5 was mediated by the receptor and not by other receptor-activated kinases. Therefore, we analyzed the ability of βRic to directly phosphorylate Stat5 *in vitro* in a nonradioactive phosphorylation assay. Stat5 was phosphorylated only in the presence of βRic (II, Fig. 1E). In autokinase assay from immunoprecipitated βRic (II, Fig. 1F) we could not detect other phosphorylated proteins even after longer exposures, suggesting that the phosphorylation of Stat5 was mediated directly by PDGF β-R and not by coimmunoprecipitating kinases.

2.3. The effect of kinase negative c-Src, Jak1 and Jak2 on the PDGF-induced activation of Stat5 (II)

According to our results, it seemed that the PDGF β-R phosphorylated Stat5 directly. To explore the possibility that Jak or Src kinases participate in the activation of Stat5 indirectly, we used the catalytically inactive mutants of c-Src, Jak1 and Jak2 kinases. These mutants, when coexpressed, compete with and inhibit wild type kinases and may also overcome the redundancy of the different Src and Jak family members (Briscoe *et al.*, 1996; Kohlhuber *et al.*, 1997). The effects of Src-KN (Lys297Met), Jak1-KN (Lys835Glu), and Jak2-KN (Lys882Glu) on the PDGF-induced tyrosine phosphorylation and DNA-binding activity of Stat5 were analyzed. Coexpression of Jak1-KN or Jak2-KN had no inhibitory effect on the PDGF-induced phosphorylation or DNA-binding of Stat5, while Src-KN inhibited the PDGF-induced activation of Stat5 (II, Figs. 5A and 5B). Interestingly, c-Src inhibited the activation to the same degree as the Src-KN, while no inhibition was observed in cells cotransfected with wild-type Jak1 (II, Fig. 5C). The observed inhibition by Src-KN could either result from abrogation of Src kinase activity or from competition between Src

and Stat5 for binding to the receptor. According to our results, it was quite obvious that the inhibition was due to the competition between Src and Stat5. We also utilized $Src^{-/-}$ and $Fyn^{-/-}$ cells to further analyze if the PDGF-induced activation of Stat5 was dependent on either one of these PDGF β-R-activated Src kinases. However, tyrosine phosphorylation of Stat5 occurred at similar levels and kinetics in $Src^{-/-}$ and $Fyn^{-/-}$ cells as in normal control fibroblasts, indicating that both c-Src and Fyn are dispensable for the PDGF-induced activation of Stat5 (II, Fig. 6).

These results indicate that the catalytic activity of c-Src, Jak1 or Jak2 is not required for the PDGF-induced activation of Stat5. Together with the results from receptor mutant experiments, the results further suggest that Stat5 and c-Src compete for the same receptor binding sites, which results in reduced phosphorylation of Stat5.

3. p100 functions as a coactivator for Stat5 in HC11 cells (III)

PRL and glucocorticoid hormones act synergistically to induce the β-casein gene expression in mammary epithelial cells. The known regulatory factors governing milk protein gene expression include Stat5, GR, C/EBPs, NF-1, YY1 (reviewed in Rosen *et al.*, 1998), and CBP/p300 (Pfitzner *et al.*, 1998). Of these factors, Stat5 has been shown to directly interact with GR (Stocklin *et al.*, 1996), C/EBP (Wyszomierski and Rosen, 2001), CBP/p300 (Pfitzner *et al.*, 1998), and YY1 (Bergad *et al.*, 2000). These interactions might complement regulatory mechanisms, which are based on the composite structure of promoters with respect to transcription factor binding sites. Additionally, they bring into proximity the binding sites for Stat5 and other transcription factors possibly to promote their functional interactions.

The p100 coactivator was first identified in HeLa cells by its ability to interact with an Epstein-Barr virus-encoded transcription factor, Epstein-Barr virus nuclear antigen-2 (EBNA-2), and a component of the transcription initiation complex, transcription factor IIE (Tong *et al.*, 1995). Coactivation was further demonstrated by transfection of p100 into B-lymphoblasts, where it enhanced the transactivational activity of EBNA-2. Analysis of the p100 sequence revealed four repeated domains that have similarity to staphylococcal nucleases (SN-like domains) (Callebaut and Mornon, 1997; Ponting, 1997a). The similarity suggested that p100 shares a common protein fold with staphylococcal nucleases, although key residues required for nuclease activity were absent. A domain near the C-terminus, a TD domain, which is also found in multiple copies in the Tudor protein of *Drosophila* (reviewed in Ponting, 1997b) was also identified. Interestingly, when proteins that play a role in controling lactation were sought, one of these proteins was identified as p100 (Broadhurst and Wheeler, 2001). In addition, the p100 protein was earlier shown to be present in endoplasmic reticulum and in lipid droplets of milk-secreting cells, and to be localised to both the membrane/organelle fraction and the nuclei of mammary epithelial cells (Keenan *et al.*, 2000). Interestingly, the p100 protein levels were increased in response to lactogenic stimuli in mammary cells. However, the function of this coactivator in mammary epithelial cells remained to be demonstrated.

3.1. p100 enhances the transcriptional activity of Stat5 in HC11 cells

At the beginning of this study, we became interested in finding proteins interacting with Stat5. At the same time in our laboratory, Dr. Yang Yie found that a coactivator protein p100 interacted with Stat6 and functioned as a coactivator in Stat6-dependent transcriptional regulation. When, in addition, p100 protein was found to be abundant in the nuclei of mammary epithelial cells (Broadhurst and Wheeler, 2001), and the protein levels were shown

to be increased in response to lactogenic hormones during lactation and to correlate well with the induction of the β-casein gene expression, we started to consider whether p100 is involved in the PRL-induced transcriptional activation of Stat5. Mouse HC11 mammary epithelial cells were used for most of our studies, since they had earlier been used to characterize molecular events involved in the regulation of milk protein expression, and also the increase in p100 protein levels was demonstrated in these cells. Treatment of HC11 cells with lactogenic hormones insulin, glucocorticoids and PRL results in transcription of the β-casein gene (Ball *et al.*, 1988; Doppler *et al.*, 1989).

Broadhurst and coworkers suggested that the modulation of p100 activity in mammary gland might occur through post-transcriptional control of expression, since the increase in p100 protein abundance occurred without a corresponding increase in p100 mRNA (Broadhurst and Wheeler, 2001). However, the role of different lactogenic hormones in up-regulation of p100 protein was not investigated. To study this phenomenon in more detail, we generated an HC11 cell line stable expressing Flag-tagged p100 protein and a control cell line with an empty pCIneo vector. Stimulation with Dexamethasone (Dex) alone did not affect the p100 protein levels in HC11-p100 cells, while PRL stimulation increased the amount of p100 protein (Fig. 1). Stimulation with both PRL and Dex did not further affect the p100 levels when compared to PRL stimulation. In conclusion, the up-regulation of p100 protein levels is mediated by PRL signaling, and Dex is not involved in this regulation.

We next wanted to investigate whether p100 is involved in the transcriptional regulation exerted by Stat5. For this purpose, reporter gene assays were carried out in different HC11 cell lines with β-casein promoter driven reporter genes. Cooperation between Stat5 and GR is required for the β-casein expression (Stoecklin *et al.*, 1996; 1997), and accordingly, in control cells, the stimulation with PRL and Dex resulted in four-fold higher induction of the β-casein reporter gene compared to the PRL stimulation (Fig. 2A). Interestingly, in HC11-p100 cells, stimulation with PRL and Dex resulted in a two-fold higher induction compared to the control cells, suggesting that p100 increases the transcriptional activity of Stat5. We further assessed the specificity of p100 action and found that the expression of p100 did not affect the activity of NF-κB or C/EBPβ luciferase construct (Figs. 2D and 2E), indicating that the effect of p100 is targeted to Stat5 activation.

The half-palindromic GR binding sites at the β-casein gene promoter recruit GR molecules to the promoter (Lechner *et al.*, 1997a; 1997b). We next studied whether the effect of p100 on Stat5-mediated transcription was dependent on the DNA-binding of GR by using serine protease inhibitor (Spi) 2.1-reporter plasmid containing only the Stat5 response element of the promoter. We found that the stimulation with both PRL and Dex enhanced the activity of the promoter and the stimulatory effect of p100 (Fig. 2C). Since the Spi-luc2 reporter construct lacked binding sites for GR, it was likely that the effect of Dex stimulation is mediated through a physical protein-protein interaction between Stat5 and GR, as previously reported (Stoecklin *et al.*, 1997; Aittomaki *et al.*, 2000). Taken together these results, the enhancing effect of p100 on the transcriptional activity of Stat5 is not dependent on the DNA-binding of GR, but simultaneous activation of GR enhances the stimulatory effect of p100 possibly through Stat5-GR protein complex formation.

3.2. p100 does not affect the early activation events or the dephosphorylation rate of Stat5

We next considered at which state of the Stat5 activation-inactivation cycle p100 exerts its effects. The first activation steps include Stat5 phosphorylation, nuclear localization and DNA-binding. p100 might also affect the signal down-regulation through modulating the rate of Stat5 dephosphorylation. In normal physiological conditions the activation of individual Stat proteins usually lasts from a few minutes to several hours. We used

stimulations from 20 min to 20 h to see the effect of p100 during the activation-inactivation cycle. Stat5 was rapidly activated, while the inactivation occurred more slowly. The expression of p100 did not affect the tyrosine phosphorylation of Stat5 at any time point (Fig. 3A), nor did it affect the DNA-binding activity of Stat5 (Fig. 3B). These results indicate that p100 does not affect the early activation events or the rate of dephosphorylation of Stat5.

3.3. Stat5 interacts with p100 *in vivo* and *in vitro*

Since p100 had a stimulatory effect on the transcriptional activity of Stat5, we wanted to determine whether p100 functioned as a coactivator for Stat5 and interacted physically with Stat5. We investigated the potential interaction between p100 and Stat5 in coimmunoprecipitation experiments with transiently transfected HC11 cells. As a result, a stimulation-independent interaction between p100 and Stat5 was found (Fig. 4A). We then characterized the important structural features for this interaction. The SH2 domain is an essential element for the activation and function of Stat, docking the protein to tyrosine phosphorylated receptor subunits and dimerizing the phosphorylated Stat proteins. In our studies, p100 was found to associate also with the SH2 mutant of Stat5A (Fig. 4B). Thus, the interaction between p100 and Stat5 is independent of the functional SH2 domain of Stat5. The TADs of different Stats have been reported to interact with various coactivator proteins, and the Stat5-TAD was earlier shown to associate with p300/CBP (Pfitzner *et al.*, 1998). Stat5A-TAD, produced as a glutathione-S-transferase (GST) fusion protein, was found to bind to p100 in our assays (Fig. 5C). We used Flag-tagged expression constructs consisting of SN-like domains or TD domain of p100 to characterize the important regions of p100 for the interaction. In addition to the full-length p100, Stat5 was found to coimmunoprecipitate with both the TD domain and SN-like domains of p100, suggesting that both regions mediate association with Stat5 (Fig. 4C). Consistent with the *in vivo* results, also the GST fusion proteins of TD and SN-like domains bound Stat5 (Fig. 5A). Our results show that p100 functions as a coactivator for Stat5, since it interacts with Stat5 and stimulates the transcriptional activity of Stat5. Stat5-TAD and the SN-like and TD domains of p100 mediate this interaction.

4. Pim-1 inhibits Stat5 activity (IV)

A Stat5 target gene *pim-1* encodes a serine/threonine kinase that has been found to fuction as a survival factor in myeloid cells deprived of IL-3 (Lilly *et al.*, 1999). In addition, Pim-1 has been linked to cell mitogenesis and proliferation and to the inhibition of apoptosis induced by genotoxic stress (Lilly and Kraft, 1997). The expression of Pim-1 is induced by several cytokines including IL-2, IL-3, GM-CSF, EPO, and PRL (Lilly *et al.*, 1992; Lilly and Kraft, 1997). Interestingly, it has been proposed that the Stat5-mediated IL-3 response may be processed by the products of the Stat5 target genes c-*fos* and *pim*-1 (Mui *et al.*, 1996).

SOCS-1 was first identified as an autofeedback inhibitor of Jaks (Endo *et al.*, 1997). As a potent inhibitor of Jaks, the levels of SOCS-1 protein must be tightly regulated. The exact mechanism by which the SOCS-1 protein is degraded is not well understood, and studies on the interaction between SOCS-1 and Elongin BC have suggested both a role for Elongin BC complex in targeting SOCS-1 to the proteasomal degradation pathway (Zhang *et al.*, 1999a), and also provided evidence that Elongin BC complex helps to stabilize SOCS-1 (Kamura *et al.*, 1998). Recently, all three members of the Pim family of kinases were shown to bind and phosphorylate SOCS-1 (Chen *et al.*, 2002). Phosphorylation of SOCS-1 by the Pim kinases prolonged the half-life of SOCS-1 and potentiated the inhibitory effect of SOCS-1 on Jak-Stat activation. These data suggested that Pim kinases might regulate Stat signaling by modulating SOCS-1 protein levels.

4.1. Pim-1 inhibits Stat5 activation, but Stat5 is not a direct substrate for Pim-1

Pim-1 can stimulate activities of several transcription factors, such as c-Myb and Nuclear Factor of Activated T Cells c (NFATc), and we hypothesized that it might be possible for Pim-1 to regulate also the activity of Stat5 (Leverson *et al*., 1998; Rainio *et al*., 2002). To examine this possibility, we used IL-3-dependent murine myeloid FDCP1 cells stably expressing neomycin (FDCP1/Neo) or 44 kDa Pim-1 protein (FDCP1/Pim44) as a cell model. In luciferase assay with transiently transfected Stat5-dependent luciferase reporter construct, the activity of Stat5 was inhibited by about 50% in FDCP1/Pim44 cells as compared to FDCP1/Neo cells after IL-3 stimulation (Fig. 1A), suggesting that Pim-1 kinase had an inhibitory effect on Stat5 activity. The expression of the kinase-deficient Lys67Met Pim-1 mutant returned the activity of Stat5 in FDCP1/Pim44 cells at the levels seen in FDCP/neo cells (Fig. 1B), suggesting that the kinase activity of Pim-1 is required for inhibition of Stat5 activity. By contrast, the 33 kDa wild-type Pim-1 kinase further enhanced the inhibitory effects of the 44 kDa Pim-1, implying that the two isoforms of Pim-1 can inhibit Stat5 activity in a dose-dependent fashion (Figure 1B).

To demonstrate that the negative effects of Pim-1 were specific for the Stat5-dependent reporter and not a result of some general inhibition, we tested also luciferase constructs containing multiple NFAT or AP-1 sites in this same cell model. As previously observed in Jurkat T-cells transiently expressing Pim-1 protein (Rainio *et al.,* 2002), also in FDCP1 cells Pim-1 was able to enhance NFATc-dependent transactivation, while AP-1 activity was only slightly affected (Fig. 1C). These results indicated that the effect of Pim-1 on luciferase reporter activities specifically depended on the transcription factors involved. Transactivation assays were carried out also with transiently transfected HC11 cells stimulated with PRL and HeLa cells stimulated with EPO (Fig. 2 A and B). Ectopic expression of Pim-1 inhibited the activity of Stat5 in both cell lines, implying that the observed inhibition is not specific for IL-3 signaling or FDCP1 cells only, but that Pim-1 is capable of inhibiting Stat5 activation also in other signaling pathways.

As Pim-1 inhibited the transcriptional activity of Stat5, we next studied whether Pim-1 inhibited the early activation events of Stat5 after IL-3 stimulation. The DNA-binding activity of Stat5 was lower in FDCP1/Pim44 cells as compared to FDCP1/Neo cells (Fig. 3A). Also the degree of tyrosine phosphorylation of Stat5 was reduced in FDCP/Pim44 cells (Fig. 3B). These data indicate that Pim-1 inhibits the early activation events of Stat5.

Tyr694 is required for the activation of Stat5, but also phosphorylation on Ser725 and Ser779 have been observed (Reddy *et al.*, 2000). However, the precise function of Stat5 serine phosphorylation remains unknown. Since Pim-1 is a serine/threonine kinase, one possible mechanism for observed inhibition would be the phosphorylation of Stat5 by Pim-1. In *in vitro* kinase assays GST-Pim-1 fusion protein was not able to phosphorylate a GST fusion protein of Stat5-TAD containing both Ser725 and Ser779, or the full-length Stat5 protein expressed in COS-7 cells (Fig. 4A, and data not shown). In coimmunoprecipitations from transiently transfected COS-7 cell lysates, Stat5 interacted with the positive control p100 as expected (Paukku *et al*., in press), but did not interact with Pim-1 (Fig. 4B). Similarly, the coimmunoprecipitations carried out from FDCP1 cell lysates showed no interaction between Pim-1 and Stat5 (data not shown). According to these results, Pim-1 does not bind to Stat5 or phosphorylate Stat5.

4.2. SOCS-1 and SOCS-3 cooperate with Pim-1 to inhibit Stat5 activity

Since Pim-1 was able to inhibit Stat5 tyrosine phosphorylation in our studies, and Stat5 was not a direct substrate for Pim-1, we hypothesized that another protein must be responsible for mediating this effect. According to a recent publication, where Pim-1 was found to function through SOCS-1 (Chen *et al*., 2002), it was possible that the mediator of

inhibition could be a member of the SOCS family. To investigate this possibility, the effect of SOCS-1, SOCS-2 and SOCS-3 on Stat5 activity was tested in FDCP1/Neo and FDCP1/Pim44 cells in luciferase assay. All three SOCS proteins were able to inhibit the IL-3-stimulated activity of Stat5 in a dose-dependent fashion in FDCP1/Neo cells (Fig. 5, and data not shown). However, SOCS-1 and SOCS-3 appeared to be more efficient inhibitors than SOCS-2. Furthermore, only these two SOCS family members were able to synergize with the enforced expression of Pim-1 in FDCP1/Pim44 cells (Fig. 5), resulting in a more profound inhibition. These results suggest a specific cooperation between Pim-1 together with SOCS-1 and SOCS-3.

Even though Pim-1 and SOCS-3 synergized in inhibiting Stat5 activation, the effect may still be indirect. It has been demonstrated that Pim-1 phosphorylates SOCS-1 and stabilizes the protein (Chen *et al.*, 2002). Because of the high homology between SOCS-1 and SOCS-3, it was likely that also SOCS-3 could function as a substrate for Pim-1. Therefore we investigated the possible interaction between SOCS-3 and Pim-1 and the ability of Pim-1 to phosphorylate SOCS-3. Our results from coimmunoprecipitation assay showed that Pim-1 indeed interacted with SOCS-3 (Fig. 6A). In addition, wild-type Pim-1 was able to phosphorylate SOCS-3 in *in vitro* kinase assay, whereas the kinase-deficient mutant Pim-1 was not (Fig. 6B). These results thus suggest that SOCS-3 is a direct substrate for Pim-1.

DISCUSSION

1. PDGF-induced Stat5 activation

A large number of SH2 domain-containing proteins have been shown to bind to the activated PDGF α- and β-Rs. We showed that the stimulation of Swiss 3T3 cells with PDGF leads to tyrosine phosphorylation of Stat1, Stat3, and Stat5 (I). The DNA-binding activity of Stat6 was detected in NIH3T3 cells, while activation of Stat2 or Stat4 was neither detected in Swiss 3T3 cells nor in PAE cells. The Stat-dependent biological signals transduced by the PDGFRs are still unknown. The signaling components and receptor amino acid residues critical for this study are presented in Figure 11.

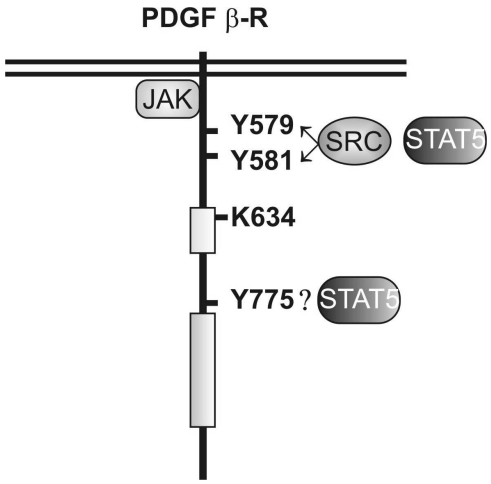

Figure 11. The intracellular part of the PDGF β-R. The critical cellular components and amino acid residues of the receptor related to this study are presented. K634 is the critical residue for receptor kinase activity, Y579 and Y581 are binding sites for Src and Stat5, and Y775 may also be involved in the binding of Stat5.

1.1. Role of Src in the PDGF-induced activation of Stat5

We became interested in studying the activation mechanism of Stat5 by PDGF β-R. At the time we started these studies, it was known that Jak and Src kinases become activated following PDGF stimulation, but the exact function of these kinases at RTKs remained unknown. It was known that Src family kinases are transiently activated by a number of growth factors and play important roles in DNA synthesis (Abram and Courtneidge, 2000), but little was known about the components of the signaling cascade initiated by Src at RTKs. Interestingly, v-Src was described to associate with Stat3 in v-Src-transformed cells and to be able to phosphorylate and activate Stat3, and Stat5 was also found to be constitutively active in v-Src-transformed cells (Yu *et al.*, 1995; Cao *et al.*, 1996; Chaturvedi *et al.*, 1997). Thus, Src was a candidate kinase for tyrosine phosphorylation of Stat5. In our studies, we found c-Src to be very active in the autokinase assay but a poor activator of Stat5 (II). Taken together, our results did not support a direct c-Src-mediated activation mechanism of Stat5 (II). This was in line with other studies showing that c-Src, even when highly overexpressed, was a considerably less efficient activator of Stat3 than v-Src (Yu *et al.*, 1995). In addition, in conditions under which Stat3 coimmunoprecipitated with v-Src, no interaction between v-Src and Stat5 could be demonstrated (Chaturvedi *et al.*, 1997), thus Src may be a better activator for Stat3 than Stat5. Several studies about whether or not Src activates Stat1, 3 and 6 at the PDGFR have been published. According to Wang and coworkers, PDGF-induced Stat3

activation is dependent on Src activation, since a specific Src kinase inhibitor inhibited Stat3 activation following PDGF stimulation (Wang *et al.*, 2000c). On the other hand, Sachsenmeier and coworkers have presented that the activation of Stat1, 3 and 6 is independent of Src, because the inhibition of Src binding to PDGFR did not abolish the activation of these Stats (Sachsenmaier *et al.*, 1999). In contrast, Cirri and coworkers have suggested that Src activates both Stat1 and Stat3 at the PDGFR (Cirri *et al.*, 1997).

Although Src was not responsible for the PDGF-induced phosphorylation of Stat5, it could indirectly influence the ability of PDGF β-R to phosphorylate Stat5. Mutation of the c-Src phosphorylation site Y934 at the PDGF β-R (Hansen *et al.*, 1996) did not affect the activation of Stat5 (S. Valgeirsdóttir, unpublished data). In addition, our results obtained with fibroblasts deficient in either c-Src or Fyn showed that the PDGF-induced activation of Stat5 is independent of these Src kinases. In these experiments we could not completely exclude the possibility of redundancy in the use of different Src kinases by PDGF β-R; triple knockout mice deficient in c-Src, Fyn, and c-Yes would have been needed to address this question.

For several receptors mediating Stat5 activation, phosphorylation of specific tyrosine residues have been shown to be necessary for the activation of Stat5 probably by creating binding sites for the SH2 domain of Stat5 (Lebrun *et al.*, 1995; Lin *et al.*, 1995; Demoulin *et al.*, 1996; Friedmann *et al.*, 1996; Gobert *et al.*, 1996; Quelle *et al.*, 1996). Tyrosine residues 579 and 581 were found to be important for tyrosine phosphorylation and DNA-binding activity of Stat5 (I). Y579 and Y581 are located in the juxtamembrane domain of the β-R and have been shown to serve as binding sites for Src family kinases. Our experiments revealed that the SH2 domain of Stat5 was critical for the activation of Stat5 by PDGF β-R. These results suggested that inhibition of the phosphorylation of Stat5 by Y579F and Y581F mutations is most probably due to the decreased association of the SH2 domain of Stat5 to these mutant receptors. Stat5 was retained by immobilized tyrosine phosphorylated receptor peptides corresponding to Y579 and Y581, but not to other sites, suggesting that Stat5 may bind directly to these sites. The mutation of either Y579 or Y581 in the receptor did not affect the ability of the receptor to phosphorylate the Stat5 Y694 site *in vitro* or the catalytic activity of PDGF β-R. The expression of Src-KN resulted in decreased phosphorylation and DNA-binding of Stat5 in response to PDGF; however, similar inhibition was also seen by overexpression of the wild-type c-Src. From our results, we conclude that Stat5 binds to PDGF β-R tyrosines Y779 and Y781 and that the activation of Stat5 is independent of Src but that Stat5 and Src compete for the same binding sites at the receptor. Similarly, Sachsenmaier and coworkers have shown that while the activation of Stat1 and Stat3 is dependent on Y579 and Y581, the activation of these Stats is not dependent on Src activation (Sachsenmaier *et al.*, 1999).

1.2. Role of receptor tyrosine 775

Also the mutation of Y775 affected the activation of Stat5 (I). Y775 is located in the kinase insert and has been shown to participate in the binding of the small adaptor proteins Grb2 and Grb7 to the β-R (Yokote *et al.*, 1996). Y775 is not conserved in the α-R, and it is possible that this explains the difference we saw between Stat5 tyrosine phosphorylation between α- and β-Rs, β-receptor being a more efficient inducer for Stat5 activation (I). The finding that a chimeric PDGF β-R, in which the endogenous kinase insert was replaced with the corresponding stretch from the α-R, showed a considerable reduced Stat5 phosphorylation in response to PDGF stimulation, supported this notion. Also, Y775 appeared to have the weakest affinity for Stat5 based on the phosphopeptide competition data, whereas a mutation of this residue in the receptor led to almost equal decrease in the tyrosine phosphorylation of Stat5 as in the case of the Y581 mutant. It is possible that even though Stat5 binds to

phosphorylated Y579 and Y581 with a higher affinity than Y775, a mutation of Y579 might partly be compensated by Y581 and *vice versa*, making the effect on tyrosine phosphorylation of Stat5 weaker, as compared to a singly located mutation of the Y775. We did not further study the role of Y775 in binding of Stat5 and the function of Y775 in this respect remains open.

1.3. Jaks at PDGFR

We showed complex formation between the PDGF β-R and Jak1, Jak2, and Tyk2 (I). The PDGF α-R formed a less stable complex with Jak1, whereas complex formation between the α-R and Jak2 or Tyk2 was not detected. Jak1 was also tyrosine phosphorylated in response to PDGF-BB, whereas tyrosine phosphorylation of Jak2 was not detected in PAE cells. In cytokine receptor signaling a wealth of evidence supports a crucial role for Jak kinases in the activation of Stats. While the requirement of kinase activity of PDGFR is evident, the role of Jaks in activation of the Stat proteins upon PDGF stimulation has remained more controversial. Our results suggest that Jaks are not essential to the PDGF-induced activation of Stat5. Although Jak1 phosphorylated and activated Stat5, expression of the kinase negative mutants of Jak1 or Jak2 did not have any effect on the PDGF-induced activation of Stat5. Studies in cells defective in expression of specific Jaks, but transfected with PDGF or EGF receptors, have indicated that the activation of Stat1 and Stat3 by these receptors is not dependent on individual Jaks (Leaman *et al.*, 1996; Vignais *et al.*, 1996). On the other hand, the PDGF-induced activation of Stat3 was reported to require Jak kinase activity, whereas the activation of Stat1 was independent of Jak activity (Choudhury *et al.*, 1998; Vignais and Gilman, 1999).

The function of Jaks in EGFR signaling has been intensively studied. A direct interaction between ErbB1 and ErbB2 and Jaks has been detected (David *et al.*, 1996). Despite the fact that Jaks are apparently not necessary for Stat activation by EGFRs, it has been proposed that they may fulfill other important roles in EGFR signaling. Jaks may serve as adaptors for Stats, enabling interaction with EGFRs. The possibility of indirect coupling of Stats to EGFRs via constitutively associated Jaks is consistent with the observation that autophosphorylation sites on EGFR are not required for the Stat activation (David *et al.*, 1996). It has also been proposed that because Jaks can be phosphorylated on multiple tyrosine residues, they can provide docking sites for phosphotyrosine-binding signaling proteins, which, in turn, may serve as substrates for Jak kinases. In addition to Stat activation, Jaks have been implicated in the phosphorylation of Shc and the insulin-regulated substrate 1 and 2 (IRS 1/2) signaling molecules (Johnston *et al.*, 1995; VanderKuur *et al.*, 1995) and in the activation of Raf1 and the MAP kinase pathway in response to IFNβ, IFNγ, and OSM (Sakatsume *et al.*, 1998; Stancato *et al.*, 1998). Immunoprecipitates of Jak1 from IFNβ-, IFNγ-, and OSM-stimulated cells contained multiple tyrosine-phosphorylated proteins, giving confidence to the hypothesis that Jaks may have a function as scaffold molecules. Whether Jaks serve as docking sites for phosphotyrosine-binding signaling proteins at the PDGFR or whether they have some other important functions, remains to be determined.

1.4. Direct activation of Stat5 by PDGF β-R

Our results indicat that the PDGF β-R readily activates Stat5 in mammalian and insect cells (II). The results with immune-complex-purified βRic, which was devoid of detectable amounts of coprecipitating kinases, confirmed that the PDGF β-R is able to activate Stat5 directly. After we finished our studies, a direct activation of Stat5 by TEL/PDGFβR and purified PDGF-βR kinase domain has been reported (Gesbert and Griffin, 2000; Wilbanks *et al.*, 2000; Beisenherz-Huss *et al.*, 2001). Accordingly, PDGF β-R has been shown to activate

Stat1 directly *in vitro* (Choudhury *et al.*, 1998), and also EGFR and InsR have been shown to activate Stat factors *in vitro* (Quelle *et al.*, 1995; Park *et al.*, 1996; Chen *et al.*, 1997). Undoubtedly several tyrosine kinase receptors have the ability to activate Stat proteins directly.

1.5. Stats in PDGF signaling

The different Stat knockout mice have clearly demonstrated that the non-redundant functions of different Stats are highly specific for particular cytokines; but the phenotypes are different from those of the PDGF-deficient mice (Leveen *et al.*, 1994; Soriano, 1994; Durbin *et al.*, 1996; Kaplan *et al.*, 1996; Meraz *et al.*, 1996; Liu *et al.*, 1997; Takeda *et al.*, 1997; Udy *et al.*, 1997). The activation of Stat1 and Stat3 by PDGF has previously been compared with the activation by IFNα and IFNγ and found to be similar (Silvennoinen *et al.*, 1993b; Raz *et al.*, 1994). An example of the possible important involvement of Stats in PDGF signaling is the regulation of c-*fos* expression through the *sis*-inducible element (SIE) by Stat1 and Stat3 (Sadowski and Gilman, 1993; Robertson *et al.*, 1995). Stat1 activation often leads to cell growth arrest and/or apoptosis (Chin *et al.*, 1996; 1997). However, since cells derived from embryos with the Stat1 gene knocked out show a normal mitogenic response to PDGF (Durbin *et al.*, 1996; Meraz *et al.*, 1996), Stat1 appears not to be important for the PDGF-induced mitogenicity. Whether other biological signals transduced by the PDGFRs are dependent on Stat1 activation, is currently unclear. It has been proposed that it may be important for EGFR to recruit Stat1 in order to balance or restrict Stat3 and/or other proliferative or survival signaling pathways by the same receptor that may potentially lead to transformation (Chin *et al.*, 1996; Fukada *et al.*, 1996; Chapman *et al.*, 1999). This notion is supported by the fact that Stat3 is activated by stimulation with oncogenic PDGF (v-sis) (Garcia *et al.*, 1997) and also in tumors with deregulated EGFR signaling. It has been suggested that Stat3 activation can serve as a growth-promoting signal in human tumors that express high levels of EGFR (Kijima *et al.*, 2002) and Stat3 may promote growth also in normal cells. Indeed, in fibroblasts stimulated with PDGF, it was recently shown that Stat3-mediated c-Myc induction is required for cell cycle progression from G_1 to S phase (Bowman *et al.*, 2001), and that Stat3 is important in PDGF-induced mitogenesis. Also in vascular smooth muscle cells, PDGF-BB-induced expression of cytoplasmic phospholipase A_2 ($cPLA_2$), which is involved in PDGF-induced cell growth, is dependent on the activation of Stat3 (Yellaturu and Rao, 2003).

In our studies, Stat5 was activated in response to PDGF to a similar degree as IL-3-induced Stat5 activation in hematopoietic cells (I). This suggested that activation of Stat5 in response to PDGF is functionally relevant and of consequence for PDGF-induced biological responses, but the precise role of Stat5 in PDGF signaling remains unknown. Both Stat5 and PDGF have been implicated in the induction of adipocyte differentiation in various models (Bachmeier and Loffler, 1997; Udy *et al.*, 1997), and it is possible that the PDGF-mediated stimulation of Stat5 is involved in this and similar biological processes. Diverse roles for Stat5 have been suggested: loss of Stat5 disrupts IL-2 signaling and results in impaired T-cell proliferation and a failure to express genes controlling cell cycle progression (Moriggl *et al.*, 1999), Stat5 has been linked to cell survival (Schwaller *et al.*, 1998; Socolovsky *et al.*, 1999; Schwaller *et al.*, 2000; Ihle, 2001), and to B-cell differentiation induced by IL-4 and IL-7 (Sexl *et al.*, 2000). In addition, activation of Stat5 by a melanoma inducing EGFR-related Xmrk kinase correlated with mitogenic signaling reflected by the cell cycle progression. It was suggested that up-regulation of *pim*-1 expression by Xmrk-activated Stat5 may be sufficient to trigger cell cycle progression (Morcinek *et al.*, 2002). One important role for Stat5 in PDGF signaling may be mediated through SOCS. PDGF has been shown to induce the expression of SOCS-3, most probably via Stat5 (Cacalano *et al.*, 2001). The PDGF-

induced SOCS-3 functions in the regulation of the Ras/MAP kinase pathway and this role is executed through negative regulation of RasGAP, which results in sustained activation of the Ras pathway. There is a wealth of evidence indicating that the maintenance of Ras activation may control proliferation. Significantly, the Ras/MAP kinase pathway can promote or inhibit cell cycle progression, depending on whether its activation is sustained (Woods *et al.*, 1997). Prolonged expression and tyrosine phosphorylation of SOCS-3 may have an important role in this response.

Stat3 and Stat5 are clearly involved in the organogenesis of the mammary gland, and there is increasing evidence that the Stat proteins are persistently activated in primary breast cancers and breast cancer-derived cell lines (reviewed in Garcia and Jove, 1998; Bromberg, 2000). The potential mechanism(s) responsible for Stat tyrosine phosphorylation in primary breast cancer specimens include the overproduction of the EGF and EGFR, PDGFR, Jaks, and c-Src, and perhaps mutations within the Stat protein (Garcia *et al.*, 1997; Garcia and Jove, 1998; Sartor *et al.*, 1997). Interestingly, the myeloproliferative phenotype, induced in mice by TEL/PDGF β-R expression, depends on the PDGF β-R tyrosines 579 and 581 (Tomasson *et al.*, 2000). It was speculated that this dependence is due to the Src binding, but in light of our results, it might also result from Stat5 binding to the receptor. Evidently, also PDGF-induced activation of Stats may mediate signals for transformation by PDGF.

2. p100 functions as a coregulator for Stat5

Several hormones and factors influence milk protein gene expression in mammary epithelial cells, but the best understood are PRL and glucocorticoids. Their signals are mediated through transcription factors, which act on the promoters of the milk protein genes. In this regard, several transcription factors including Stat5, GR, NF-kB, NF-1, and C/EBP (Raught *et al.*, 1994; Li and Rosen, 1995; Liu *et al.*, 1996; Stocklin *et al.*, 1996; Lechner *et al.*, 1997b; Geymayer and Doppler, 2000) have been studied. However, a clear overall picture of how the transcriptional control of milk protein genes is achieved throughout the gestation-lactation cycle is yet to come to light. It is probable that all transcriptional activators or coactivators that are also involved have not yet been studied in this context. The abundance of mammary p100 during the gestation-lactation cycle was shown to be specifically and closely associated with lactation. Interestingly, the increased p100 abundance during lactation appears to be a common feature among mammals. The physiological role of p100 in PRL signaling had not been earlier studied.

2.1. Functional interaction between p100 and Stat5

The findings that p100 protein levels increased during lactation and that p100 was present in lipid droplets of milk secreting cells, suggested that p100 might participate in the regulation of milk gene induction. We observed that p100 enhanced the transcriptional activity of Stat5 and interacted with Stat5, which indicated that p100 functions as a coactivator for Stat5. Association between p100 and Stat5 did not require tyrosine phosphorylation or functional SH2 domain of Stat5. Since p100 has been detected in both the cytoplasm and nucleus, the interaction could occur already in the cytoplasm, and p100 and Stat5 could translocate to the nucleus as a complex. Phosphorylation of Stats is not always required for their interactions with coregulators, and for example interaction of Stat5 with GR or Stat6 and p100 is independent of tyrosine phosphorylation of Stats (Wyszomierski *et al.*, 1999; Yang *et al.*, 2002). We found an interaction between Stat5-TAD and p100, but it is possible that also other regions of Stat5 mediate the interaction with p100.

The structure of p100 consists of four repeated domains with similarity to the staphylococcal nuclease structure SN-like domains, and a TD domain (Callebaut and Mornon, 1997; Ponting, 1997a). The TD domain of p100 is a "hybrid" SN-like domain, where a domain, which is found in multiple copies in the *Drosophila melanogaster* Tudor protein, replaces the OB-fold. Staphylococcal nucleases (SNs) are small calcium-dependent enzymes, which hydrolyze both DNA and RNA. SNs consist of two subdomains, of which the first subdomain belongs to the large oligonucleotide/oligosaccharide-binding (OB)-fold superfamily (Murzin, 1993), and the second subdomain consists of two independently folded α-helices. The SN-like domains of p100 lack the catalytic amino acids present in nucleases. We found that the SN-like domains of p100 mediate interaction with Stat5. The SN-like domains of p100 have been shown to contain EVES sequence, which is able to interact with c-Myb and inhibit its activity (Dash *et al.*, 1996). In addition, p100 interacts with Pim-1 serine/threonine kinase through a sequence located in SN-like domains, but different from EVES (Leverson *et al.*, 1998), indicating that there are several protein interaction motifs in the SN-like domains of p100. Only the SN-like domain of p100 was found to mediate the interaction with Stat6 (Yang *et al.*, 2002), but we showed that also the TD domain of p100 associates with Stat5 and can therefore function as a protein interaction domain (III). TD domains are conserved protein modules of unknown function that are often present in proteins that associate with RNA (reviewed in Ponting, 1997b). The determined structure of the TD domain of Survival of Motor Neuron (SMN) protein suggests, however, that TD domains function as protein interaction motifs and do not bind to RNA directly (Selenko *et al.*, 2001). In line with this hypothesis and our results, the TD domain of SMN protein has been found to mediate interactions with a spliceosomal Sm core protein (Selenko *et al.*, 2001), and with a small nucleolar RNA-associated protein fibrillarin (Jones *et al.*, 2001). In addition, two TD domains of Trap protein (tudor repeat associator with PCTAIRE 2) have been shown to associate with a Cdc2-related kinase PCTAIRE 2 in rat brain (Hirose *et al.*, 2000).

2.2. p100 in PRL signaling

In our assay, the proper stimulation of the β-casein promoter required the activation of GR, while the enhancing effect of p100 appeared not to require GR. Activation of GR, however, enhanced the stimulatory effect of p100 and this effect was not dependent on the DNA-binding of GR. Stat5 and GR physically interact with each other, and this protein complex may mediate a more stable interaction surface with p100 than the relatively small TAD of Stat5 alone. C/EBPβ activates β-casein expression in synergy with Stat5 and GR, and several binding sites for C/EBP have been mapped in the β-casein gene promoter (Doppler *et al.*, 1995). Also NF-κB has a binding site at the β-casein promoter, and NF-κB has been shown to negatively regulate the β-casein gene expression by inhibiting the activity of Stat5 (Geymayer and Doppler, 2000). We showed that p100 had no effect on the transcriptional activity of C/EBPβ or NF-κB, therefore demonstrating the specificity of p100 on Stat5-mediated activity.

Several mechanisms, by which p100 could modulate the transcriptional activity of Stat5, existed. The increased tyrosine phosphorylation and/or DNA-binding can stimulate transcriptional activity, as has been observed in Stat5-GR cooperation (Wyszomierski *et al.*, 1999). We did not observe any changes in the early steps of Stat5 activation by p100. Serine phosphorylation has been implicated in the regulation of Stat activity, and phosphorylation of Stat1 and Stat3 on serine within their TAD is needed for maximal IFN-induced transcriptional activation (Wen *et al.*, 1995; Zhang *et al.*, 1995; Bromberg *et al.*, 1996; Horvath and Darnell, 1996; Ng and Cantrell, 1997). In the Stat5-TAD, there are two proline-juxtaposed serine residues, which become phosphorylated in the mammary gland during gestation and lactation. Several serine/threonine kinases are activated upon PRL stimulation, but the kinase

phosphorylating Stat5 is currently unknown (Yamashita *et al.*, 2001). However, serine phosphorylation of Stat5 in the mammary gland has an inhibitory effect on the transcriptional activity of Stat5 (Yamashita *et al.*, 2001), and therefore it is unlikely that the function of p100 would be to recruit a serine kinase to Stat5. Modulation of the chromatin structure by histone acetylation is critical for the activation of transcription, and several coactivators, like p300/CBP, possess HAT activity. p100 has earlier been shown to be devoid of HAT activity (Wang *et al.*, 2000b), and the function of p100 does not appear to involve direct modulation of chromatin structure. Coactivators can also function as bridging factors to the basal transcription machinery. p100 was found to increase the IL-4-induced transcriptional activity of Stat6 possibly by bridging Stat6 to RNA polymerase II (Yang *et al.*, 2002). It is thus likely that p100 mediates a similar function in Stat5 by bridging it with the basal transcriptional machinery.

We found that p100 levels were increased after PRL stimulation. The high abundance of p100 during lactation and its ability to cooperate with Stat5 originally suggested to us that p100 might be involved in milk protein gene expression. The modulation of p100 activity in mammary epithelial cells occurs likely through post-transcriptional control of expression, since the increase in p100 protein abundance occurs without a corresponding increase in p100 mRNA (Broadhurst and Wheeler, 2001). These results together with our findings demonstrating the costimulation of Stat5-mediated transcription by p100, suggest the existence of a positive regulatory loop in PRL-induced transcription, where PRL stabilizes p100 protein, which in turn can cooperate with Stat5 in transcriptional activation of the β-casein gene promoter, and probably also in other Stat5-regulated milk gene promoters. Diverse, cell type and cytokine specific interactions between Stat factors and non-Stat transcription factors might contribute to the different phenotypic effects elicited by individual cytokines utilizing the Jak/Stat pathway.

3. Pim-1 negatively regulates the activity of Stat5

3.1. Cooperation between Pim-1 and SOCS proteins

Studies with both the transcriptionally inactive TAD deletion construct of Stat5 and the hyperactivated mutant Stat5 have shown that the expression of several genes, including *cis*, *osm* and *pim-1,* is dependent on Stat5 (Mui *et al.*, 1996). Under physiological conditions, cytokine stimulation first results in an activation phase, which is then quickly quenched by negative regulators such as CIS, SOCS, $p21^{WAF1/Cip1}$, and tyrosine phosphatases. Interestingly, Stat5 has been shown to be able to regulate both phases. SOCS proteins are encoded by immediate early genes that act in a feedback loop to inhibit cytokine responses and activation of Stats. The constitutive expression of SOCS-1 was shown to be sufficient to induce apoptosis of Ba/F3 cells, whereas constitutive expression of Pim-1 was sufficient to induce IL-3-independent growth of Ba/F3 cells (Nosaka *et al.*, 1999). These findings suggested that Stat5 might regulate cell fate by varying the intensity and duration of the expression of a set of target genes. The Pim kinases and SOCS proteins are induced by a variety of cytokines, and the interaction between Pim and SOCS may represent a mechanism by which cytokines cross-regulate one another. For example, expressions of Pim-1, Pim-2, and SOCS-1 are all induced by IFNγ (Yip-Schneider *et al.*, 1995; Starr *et al.*, 1997), and the interplay between Pim kinases and SOCS-1 may be important for IFNγ-induced inhibition of IL-4 signaling (Dickensheets *et al.*, 1999; Venkataraman *et al.*, 1999).

In our studies, we found that Pim-1 inhibits the transcriptional activity of Stat5 in IL-3, PRL, and EPO signaling, and that the kinase activity of Pim-1 is required for this inhibition. The inhibition was seen at the levels of tyrosine phosphorylation, DNA-binding and

transactivation of Stat5. However, Pim-1 did not interact with Stat5 or phosphorylate Stat5, which encouraged us to study whether another protein might cooperate with Pim-1 in the inhibition of Stat5 activity. Interestingly, SOCS-1 and SOCS-3, and to lesser extent SOCS-2, were found to further enhance the negative effects of Pim-1 on Stat5 activity. According to our results, SOCS proteins most likely mediate the observed inhibitory signal of Pim-1. We demonstrated that SOCS-3 is a direct substrate for Pim-1. In accordance with our results, SOCS-1 was earlier published to interact with Pim-1 and be phosphorylated by Pim-1 (Chen *et al.*, 2002). It is also possible that Pim-1 affects other Stats, especially since both Stat5 and Stat3 have recently been reported to be able to complement Pim-1 and Pim-2 in tumorigenesis (Mikkers *et al.*, 2002), and since Pim-1 has been reported to inhibit Stat6 activation after IL-4 stimulation in cooperation with SOCS-1 (Chen *et al.*, 2002) .

SOCS-1 was earlier shown to be stabilized by Pim-1. It is therefore possible that Pim-1 stabilizes also SOCS-3 by serine phosphorylation, and thereby potentiates its inhibitory effects. In this respect, it is interesting to note that the SOCS-3 function and stability can be regulated also by tyrosine phosphorylation. SOCS-3 is rapidly tyrosine phosphorylated after IL-2 stimulation, and also after various growth factor stimulations (Cohney *et al.*, 1999;Cacalano *et al.*, 2001). The tyrosine phosphorylated SOCS-3 can interact with RasGAP, maintain activation of ERK, and ensure cell survival and proliferation through the Ras pathway (Cacalano *et al.*, 2001). The phosphorylation was also shown to inhibit the SOCS-3-elongin C interaction and activate proteasome-mediated SOCS-3 degradation (Haan *et al.*, 2003).

Our results suggest that Stat5 may attenuate its own activation via the up-regulation of its target gene *pim*-1. This negative feedback loop may therefore regulate cell survival, proliferation, and differentiation. Because Pim-1 does not completely inhibit Stat5 activation, the observed inhibition may be a fine-tuning mechanism that maintains the Stat5 activity at an optimal level. During malignant transformation, forced activation of Stat5 may lead to activation of both proliferative genes, such as c-*myc*, and survival genes, such as *pim*-1, so that the inhibitory signal is overridden.

PERSPECTIVE

Stat5 functions as a downstream effector of cytokine and growth factor receptor signaling. Pleiotropic gene regulation by Stat5 has been implicated in cellular functions such as proliferation, differentiation, and apoptosis with relevance to processes of hematopoiesis and immunoregulation, reproduction, and lipid metabolism. Compared with normal cells and tissues, constitutively activated Stat5 has been identified in a wide variety of human cancer cell lines and primary tumors. In tumor cells, Stat5 activity is linked to persistent activity of tyrosine kinases, including Src, EGFR, and PDGFR. Persistent activation of Stat5 has been demonstrated to directly contribute to oncogenesis by stimulating cell proliferation and preventing apoptosis. Interestingly, in fatal myeloproliferation, induced in mice by TEL/PDGF β-R expression, the myeloproliferative phenotype requires PDGF β-R tyrosines 579 and 581. According to our results, the phenotype may be dependent on the activation of Stat5. The function of Stats in PDGF signaling is still largely unknown, but the consequences of Stat activation in both normal PDGF signaling as well as in malignant cells are important aspects for future studies.

As important as it is to characterize the mechanisms for Stat activation, is to clarify the specific cascades for Stat signal down-regulation. Stat activation is inhibited by dephosphorylation, but also by SOCS proteins, which can block the Stat activation by inhibiting Stat receptor binding or Jak activity. In our studies, it was found that a Stat5-induced protein Pim-1 could regulate the activity of Stat5 by cooperating with SOCS proteins. Interestingly, our data indicated that Pim-1 together with SOCS-1 and SOCS-3 are likely to be components of a negative feedback loop that allows Stat5 to inhibit its own activation. In the future, gene expression profiling by microarray technology is expected to reveal a molecular signature of Stat5-regulated genes and enable the subsequent studies for finding the specific functions of Stat target genes.

In this study p100 was identified as a Stat5 coactivator. Increasing knowledge of how Stat proteins affect transcriptional regulation is important as basic information for understanding the coordinated control of transcription in mammalian cells. In addition, it is necessary to know the most common interacting partners of specific transcription factors if pharmacological intervention of specific transcriptional activity is to be achieved. One of the attractive features of Stat proteins for cancer therapy is that there are only two molecular targets, Stat3 and Stat5, as opposed to a huge number of tyrosine kinases. As a matter of fact, in many cases it has been demonstrated that blocking Stat3 or Stat5 function alone is sufficient to inhibit tumor cell growth and to induce apoptosis. The interest for developing inhibitors of Stat5 for molecular-targeted cancer therapy is accelerating, as the importance of Stat5 in human cancer is becoming increasingly evident.

ACKNOWLEDGEMENTS

This work was carried out at the Department of Virology, in Haartman Institute, University of Helsinki. I wish to express my gratitude to the head of the department, professor Antti Vaheri, for excellent working facilities and for the opportunity to work at the Department of Virology. I want also thank professor Carl-Henrik von Bonsdorff for his helpful and supportive attitude.

I want to warmly thank my supervisor professor Olli Silvennoinen for his advice, patience and encouragement throughout this study. I am also grateful that somehow Olli managed to find time for me in spite of his vast workload elsewhere.

I thank professor Carl-Henrik Heldin and Dr. Sigrídur Valgeirsdóttir for collaboration. My coauthors Dr. Jie Yang, docent Päivi Koskinen, as well as MD Katriina Peltola, are also warmly thanked.

Professors Pirkko Härkönen and Lea Sistonen are thanked for their valuable criticism and comments in the preparation of this thesis manuscript.

I wish to thank warmly professor Jorma Keski-Oja for his support during this work. I also want to thank the members of Jorma Keski-Oja's lab for their friendship and help.

I thank docent Olli Ritvos for the opportunity to finish my thesis work in the Programme for Developmental and Reproductive Biology, at the Biomedicum Helsinki. I also thank people in his lab for company and help. I wish to thank Noora Kaivo-oja and Samu Myllymaa for the nice atmosphere in our shared office room.

I want to thank the members of Olli Silvennoinen's lab, Jie Yang, Marko Pesu, Ilkka Junttila, Saara Aittomäki, Paula Kosonen and Panu Kovanen, as well as the other lab members, for their friendship and helpful attitude. Pipsa Saharinen deserves my special thanks for her support and friendship, and also for sharing the lab with me during these years.

I warmly thank Tuomo Alanko, Anneli Hotti, Marko Hyytiäinen, Jouko Lohi, Eeva Martelin, Juha Saharinen, and Minna and Jussi Taipale, for their friendship and the amusing lunch and coffee breaks. In addition, many thanks to Juha for his generous help with the computers whenever needed.

I am grateful to all my friends outside the lab. Riikka Nevala is deeply thanked for our long-term friendship and countless joyful moments we have shared. I am thankful to Kirsi Piippo for her friendship and company during our numerous free time activities.

I wish to express my deepest gratitude to my parents Kirsti and Reijo Paukku and my little brother Antti for their support and love throughout my life. I also thank Antti for being such a perfect brother!

My dearest thanks go to Timo Frank for his love, support, and encouragement over the last years.

This work has been supported by the Academy of Finland, Biomedicum Helsinki Foundation, Emil Aaltonen Foundation, Finnish Cancer Organizations, Finnish Cultural Foundation, Helsinki Biomedical Graduate School, Ida Montin Foundation, Instrumentarium Scientific Fund, Maud Kuistila Foundation, the Medical Research Fund of Tampere University Hospital, Oskar Öflund Foundation, and Sigrid Jusélius Foundation.

Helsinki, September 2003

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